

## SUPPLEMENTARY INFORMATION

### Supplementary Text

#### **OueS regulates the OmpU transcriptome under virulence inducing conditions.**

OmpU is highly expressed during the infection process and is critical for intestinal colonization<sup>7,19,26</sup>. We examined the role of OmpU and OueS in the context of virulence-associated conditions. To accomplish this, we first analyzed the transcriptomes of the WT,  $\Delta ompU$  and  $\Delta ompU$ -pOmpU<sup>C6706</sup> strains under virulence-inducing (AKI) conditions<sup>62</sup>. Our analyses reveal that OmpU regulates a much larger proportion of genes under AKI conditions than during biofilm formation, modulating the expression of 947 genes (560 activated and 379 repressed), compared to 270 genes (181 repressed, 89 activated) in biofilms (**Supplementary Data Table S11**). Ectopic expression of OmpU in  $\Delta ompU$  decreases the number of differentially expressed genes to 170 from the 947 genes in  $\Delta ompU$  (82.04% recovery of the  $\Delta ompU$  transcriptome to WT levels) (**Supplementary Data Table S11**). Furthermore, most of the OmpU-modulated genes under AKI conditions are RpoE-independent as only 158 of the 947 (16.68%) are shared with the RpoE regulon under AKI conditions, suggesting a much lesser role of the envelope stress response during the infection process (**Supplementary Data Table S11**). Additionally, OueS modulates the expression of 455 of the 947 genes (48%) of the OmpU transcriptome under AKI conditions suggesting an integral role of OueS in this process (**Supplementary Data Table S12**).

## Supplementary Figures

**Figure S1. Genes regulated by OmpU during growth in biofilms.** (A) Transcriptomes of biofilm-derived cells were visualized by principal component analysis. Ectopic expression of *ompU* and OueS result in a similar shift in the datasets compared to the WT and  $\Delta ompU$  strains. n=2 biological replicate datasets for each sample. Functional enrichment of the OmpU-regulated genes was performed by the Panther Overrepresentation test (GO Biological process)<sup>102,103</sup> to classify the OmpU-repressed (B) and activated (C) datasets. OmpU primarily represses iron-related genes and activates those involved in viral defense.

**Figure S2. OueS mutants and alleles.** (A) Expression of OueS in the environmental strain of *V. cholerae* GBE1114. Size-fractionated RNA (100-500 nt) isolated from LB-grown cells were used to monitor OueS expression by qRT-PCR using primers in the conserved 3' locus of the sRNA. Expression levels in the two strains are similar and was normalized to the housekeeping gene *recA*. n=3 biological replicates. Significance was determined using unpaired t-test. The 166 ntd OueS sequence from (B) OueS 5'-mut ( $\Delta G$  -40.7), (C) OueS 3'-mut ( $\Delta G$  -24.6), and (D) OueS full length-mut ( $\Delta G$  -26.5) were used to generate their predicted secondary structures on mFold (unafold.org). The structures requiring the least free energy ( $\Delta G$ ) are shown. Mutations significantly alter the predicted structures of the OueS mutants (C, D, E), compared to the WT OueS<sup>C6706</sup> (Fig 1). (E) Sequence conservation of the *ompU* ORF encompassing the OueS 5' locus before (WT) and after codon wobble mutations (OueS 5'-mut, 3'-mut and FL-mut) are shown to demonstrate an unaltered protein coding sequence.

**Figure S3. Altered iron concentrations affect biofilm but not planktonic growth. (A)**

Biofilm formation of strains ectopically expressing the WT or mutant versions of OueS in the  $\Delta ompU$  background were examined under iron-replete (100  $\mu$ M FeCl<sub>2</sub>; dark grey bars) or iron-depleted (100  $\mu$ M 2,2'-bipyridyl) conditions, compared to the basal iron conditions (TB media; light grey bars).  $n \geq 4$  independent biological replicates. Statistical significance was determined using ordinary one-way ANOVA. The planktonic growth patterns of the WT,  $\Delta ompU$  cells ectopically expressing either the WT or mutated versions of OueS in (B) tryptone broth (baseline iron levels) and under (C) iron-replete (100  $\mu$ M FeCl<sub>3</sub>) or (D) iron-depleted (100  $\mu$ M 2,2'-bipyridyl) conditions. Growth patterns are similar between the strains in each condition irrespective of iron concentration.  $n \geq 3$  independent biological replicates. (E) Read mapping plot for the *ompU* locus based on MAPS data under iron limitation conditions.

**Figure S4. OueS<sup>C6706</sup> complements loss of ToxR under virulence-inducing conditions.**

Pairwise comparisons of AKI transcriptomes of the WT strain and the *toxR* mutants expressing alleles of OueS reveals that (A) OueS<sup>C6706</sup> restores the  $\Delta toxR$  transcriptome to closely resemble the WT, whereas (B) OueS<sup>GBE1114</sup> does not. All genes in this dataset have a *q*-value of  $\leq 0.01$  and are listed in Supplementary Table 6.  $n=2$  independent biological replicates.

**Figure S5. OueS is expressed from the *ompU* promoter. (A)**

qRT-PCR analysis using RNA isolated from AKI-grown cultures reveal that OueS expression in the  $P_{ompU}$ -OueS

strain (0.93-fold) is similar to the WT and is 8-fold higher in the strain expressing OueS ectopically from an arabinose-inducible promoter. Expression was normalized to the housekeeping gene *recA*. (C) Biofilm assays reveal that both chromosomal and ectopic OueS expression suppresses biofilm formation like the WT strain.  $n \geq 3$  independent biological replicates. Statistical significance was determined using two-tailed unpaired T-test  $p$ -value \*\*  $\leq 0.001$ , \*\*\*\*  $\leq 0.00001$ .

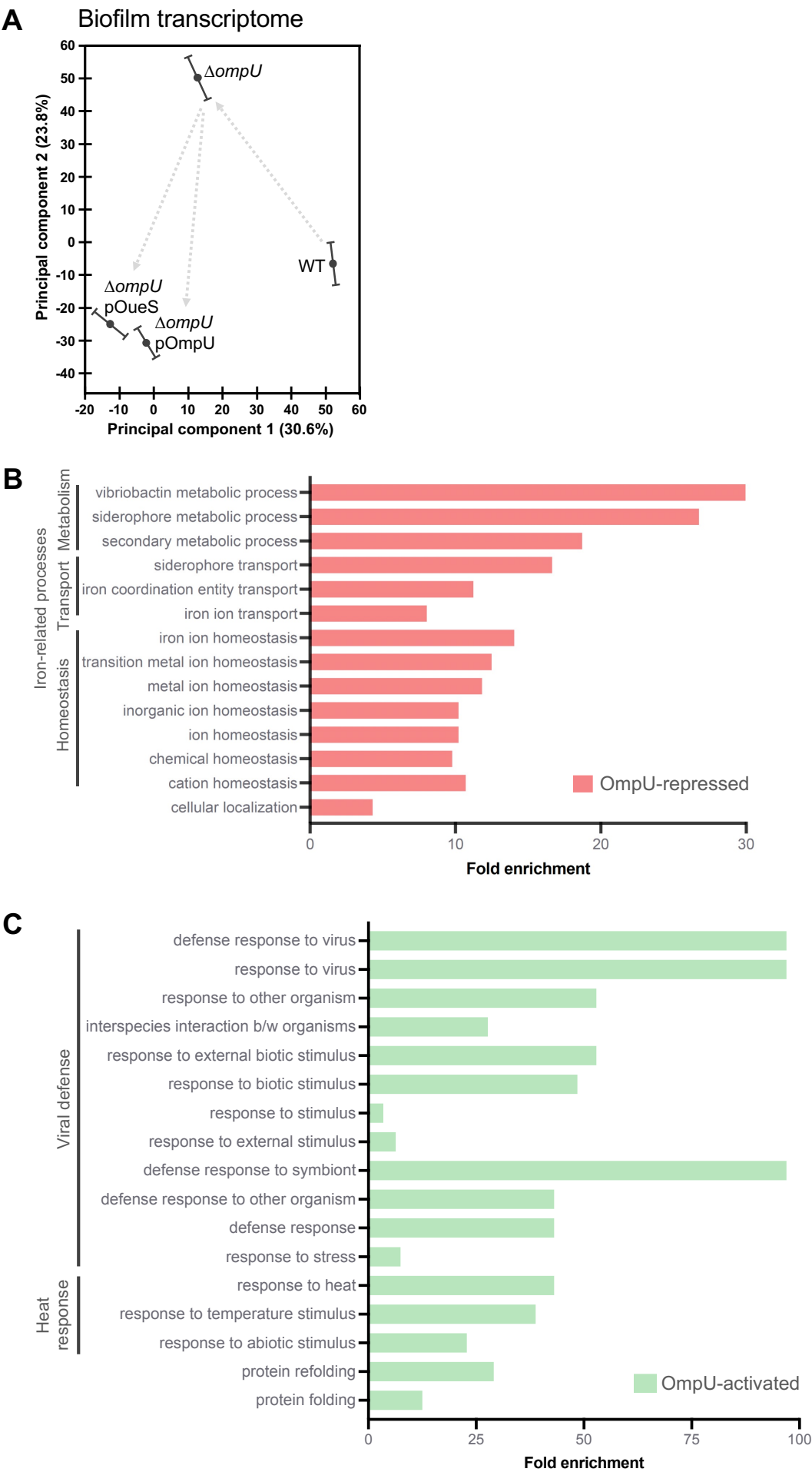
**Figure S6. Variation within the OueS alleles.** *ompU* alleles were analyzed for sequence variation<sup>41</sup> and OueS sequences were extracted from 14 representative clades. Sequence alignment of the OueS loci from representative alleles demonstrates extensive variability in the region within the *ompU* ORF but is conserved in the 3' UTR. The *ompU* stop codon is highlighted in red.

**Figure S7. Natural transformation mediates allele exchange at the *ompU* locus.** The *ompU* locus from the toxigenic C6706, the isogenic mutant encoding the environmental allele C6706::*ompU*<sup>GBE1114</sup> and three bile-resistant revertants strains isolated after transformation (Rev1 – Rev3) were sequenced and aligned using the progressive alignment algorithm. Residues that are different from the toxigenic allele are highlighted. The revertants have *ompU* sequences identical to C6706, indicating successful integration of the transformed DNA.

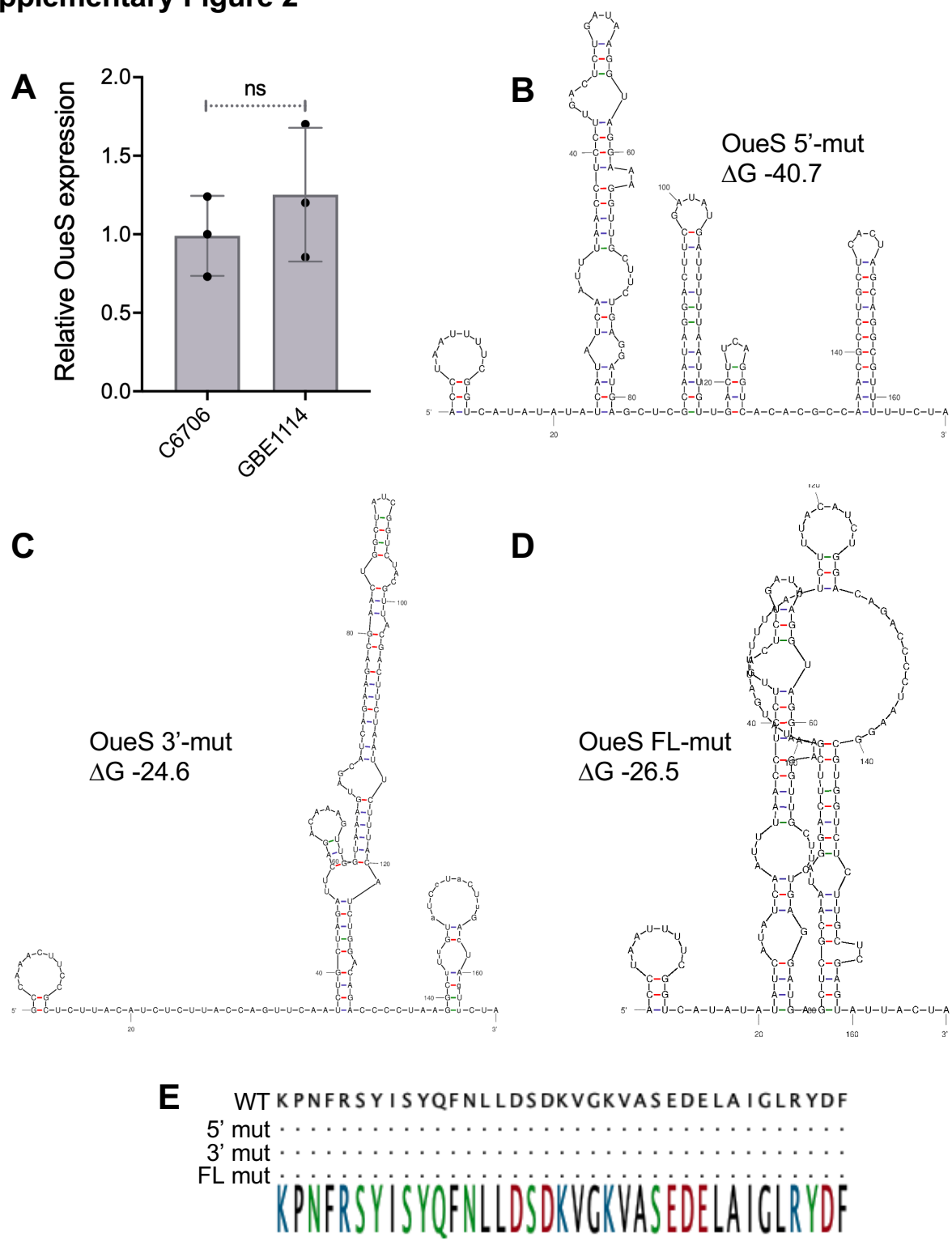
**Figure S8. Transcriptome of OueS alleles under virulence-inducing conditions.** Strains encoding a clinical-like allele (OueS<sup>GBE1194</sup>) and an environmental allele

(OueS<sup>GBE1116</sup>) were selected based on mouse intestinal colonization data (**Fig 5G**) and transcriptomes were generated under virulence-inducing AKI conditions. Comparing the transcriptomes of two clinical-like and environmental alleles reveals that 96 and 66 genes are modulated uniquely by clinical-like and environmental OueS, respectively (**Table S10**). n=2 independent biological replicates.

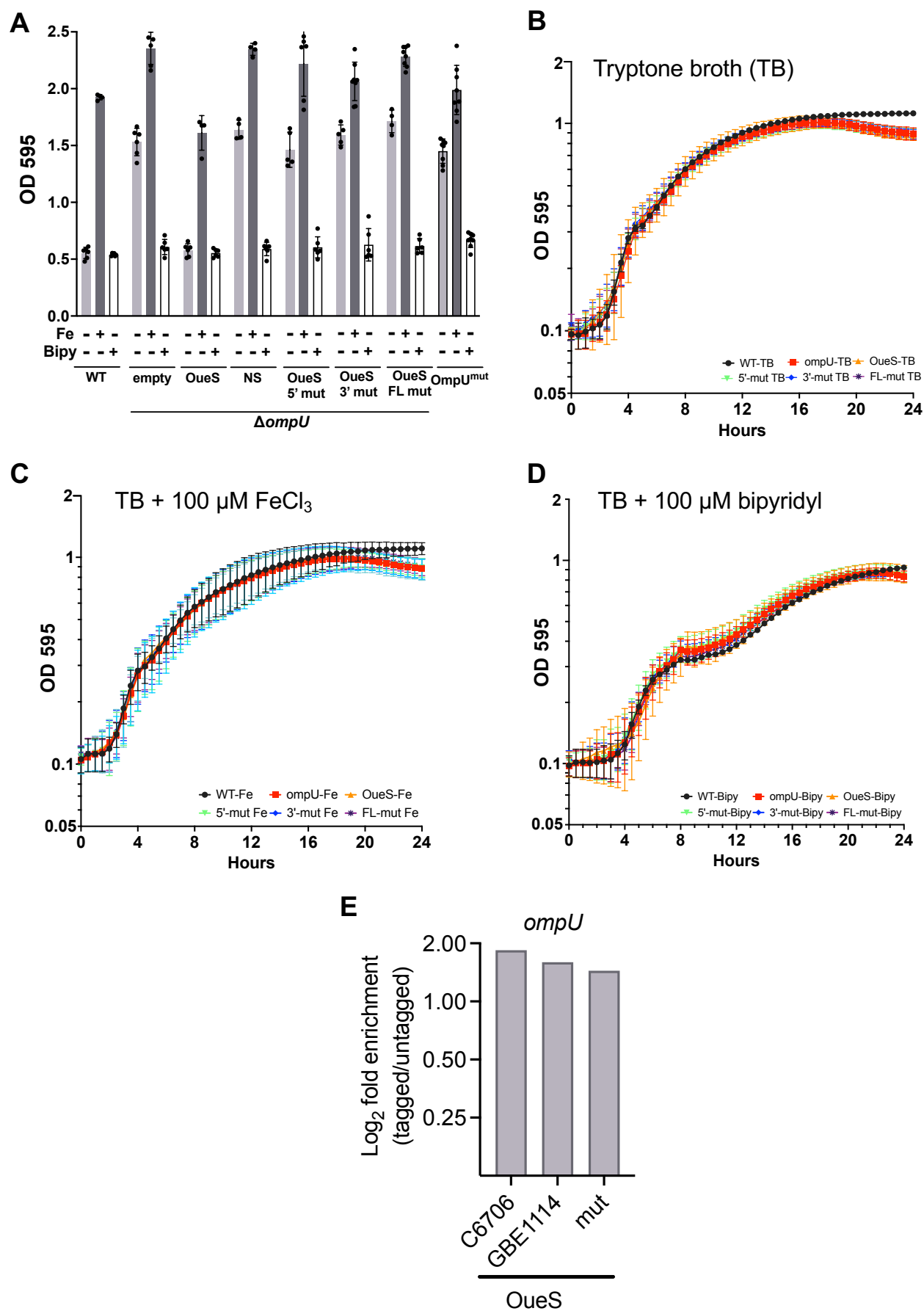
Supplementary Figure 1



Supplementary Figure 2

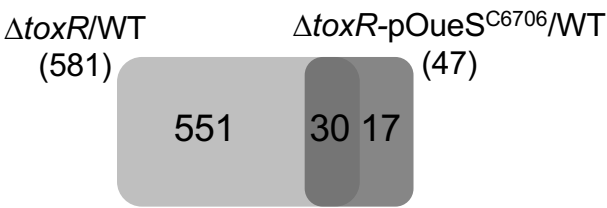


### Supplementary Figure 3

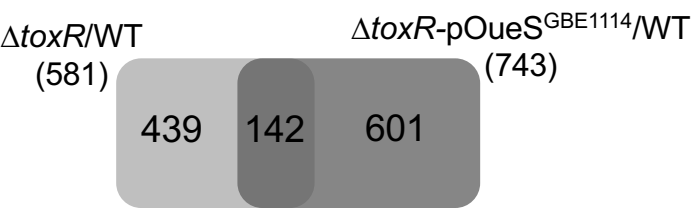


Supplementary Figure 4

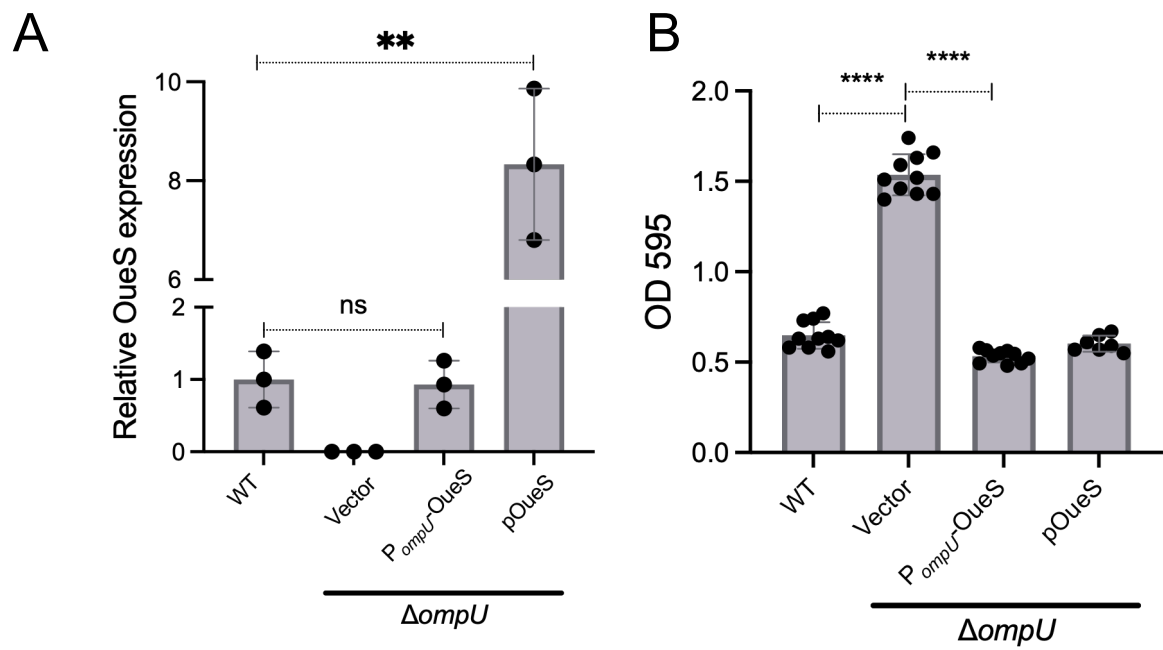
A



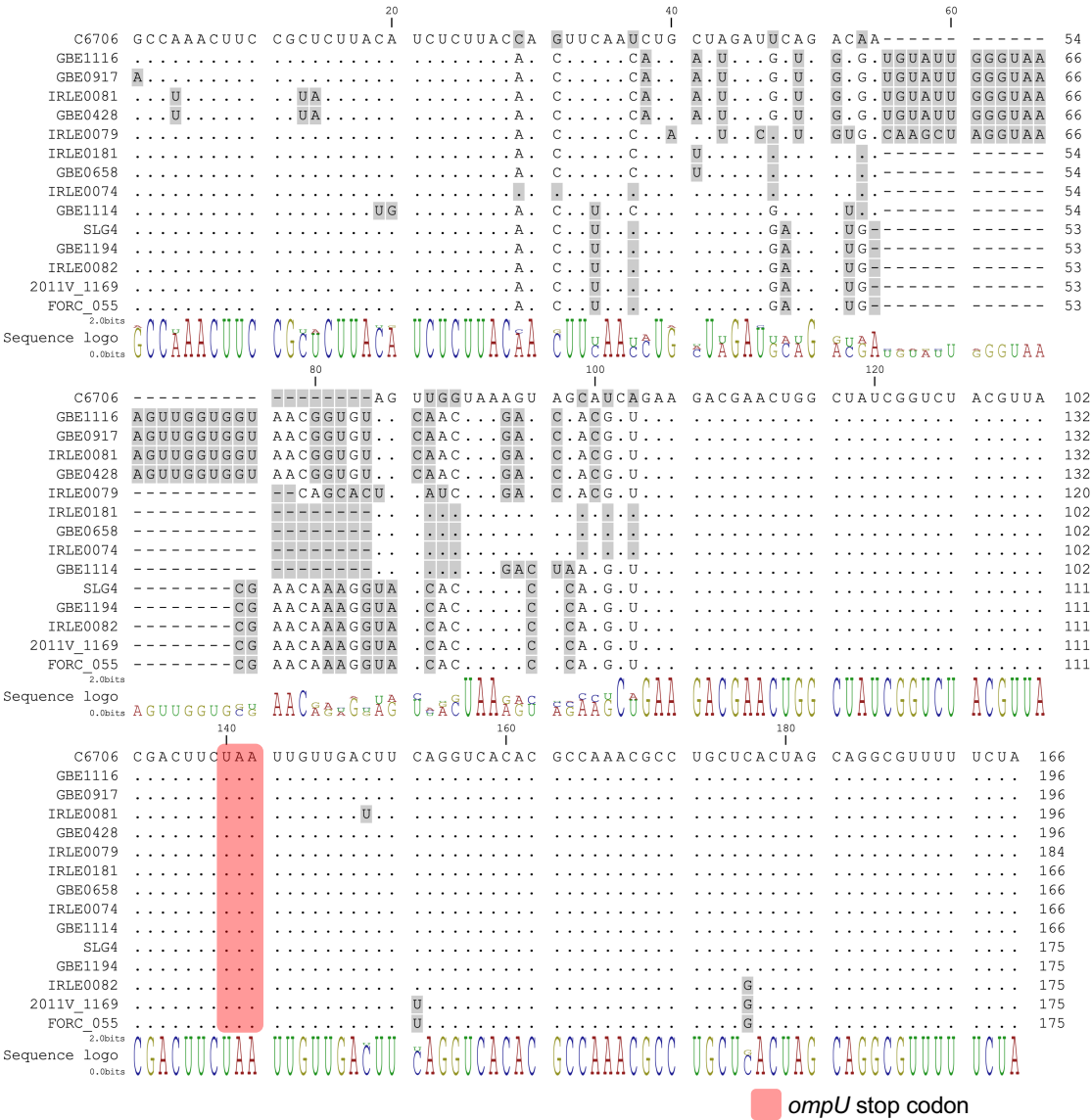
B



Supplementary Figure 5



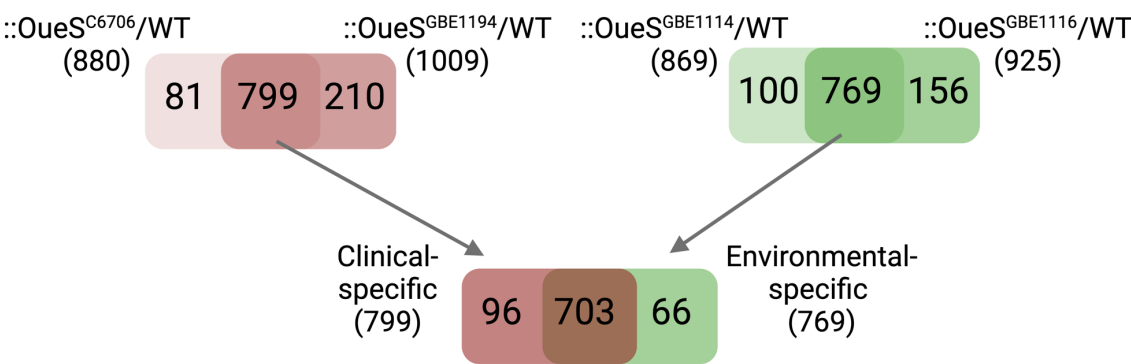
Supplementary Figure 6



## Supplementary Figure 7

[illegible]

Supplementary Figure 8



93     **Supplementary Tables**

94     Table S1: Distribution of the toxigenic OueS allele across 378 dereplicated *Vibrio*  
95     *cholerae* clades.

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Supplementary Table 1: Distribution of the toxicogenic OusS allele across 378 dereplicated *Vibrio cholerae* clades.

| accid                   | isolate                | accid | ident | length | mismatch | gapopen | qstart  | qend    | sstart | slen | eval     | bitscore | qlen    | qlen | Isolated source | Location             | Group | PCL | LPS | CTX | VP1-1 | VP1-2 M1 | VP1-2 M2 | VP1-2 M3 | VSP-1 | VSP-6 M1 | VSP-6 M2 (N16961) | VSP-6 M2 (C6766) |
|-------------------------|------------------------|-------|-------|--------|----------|---------|---------|---------|--------|------|----------|----------|---------|------|-----------------|----------------------|-------|-----|-----|-----|-------|----------|----------|----------|-------|----------|-------------------|------------------|
| GCF_000168935-Contig5   | GCF_000168935_OusS6708 | 100   | 166   | 0      | 0        | 0       | 93070   | 93175   | 166    | 1    | 6.53E-86 | 307      | 180190  | 166  | Human           | Bangladesh           | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_000221465-Contig114 | GCF_000221465_OusS6708 | 100   | 166   | 0      | 0        | 0       | 133048  | 133211  | 166    | 1    | 2.88E-86 | 307      | 180240  | 166  | Unknown         | USA                  | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_001402185-Contig25  | GCF_001402185_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87672   | 87837   | 1      | 166  | 1.25E-85 | 307      | 347746  | 166  | Human           | USA                  | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_002097735-Contig22  | GCF_002097735_OusS6708 | 100   | 166   | 0      | 0        | 0       | 92850   | 93015   | 166    | 1    | 5.12E-86 | 307      | 141180  | 166  | Human           | Mozambique           | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003347715-Contig2   | GCF_003347715_OusS6708 | 100   | 166   | 0      | 0        | 0       | 92910   | 93075   | 166    | 1    | 8.52E-86 | 307      | 180756  | 166  | Water           | Bangladesh           | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003347775-Contig162 | GCF_003347775_OusS6708 | 100   | 166   | 0      | 0        | 0       | 92821   | 92886   | 166    | 1    | 8.52E-86 | 307      | 180793  | 166  | Water           | Bangladesh           | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003348095-Contig56  | GCF_003348095_OusS6708 | 100   | 166   | 0      | 0        | 0       | 85990   | 86155   | 1      | 166  | 6.45E-86 | 307      | 178976  | 166  | Water           | Bangladesh           | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003348335-Contig16  | GCF_003348335_OusS6708 | 100   | 166   | 0      | 0        | 0       | 93013   | 93178   | 166    | 1    | 6.52E-86 | 307      | 180641  | 166  | Water           | Bangladesh           | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003348335-Contig165 | GCF_003348335_OusS6708 | 100   | 166   | 0      | 0        | 0       | 807     | 972     | 1      | 166  | 1.43E-86 | 307      | 39156   | 166  | Water           | Bangladesh           | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003348415-Contig558 | GCF_003348415_OusS6708 | 100   | 166   | 0      | 0        | 0       | 3493410 | 3493575 | 166    | 1    | 1.43E-84 | 307      | 4497081 | 166  | Human           | Indonesia            | G3    | PCL | x   | x   | x     | x        | x        | x        | x     | x        | x                 | x                |
| GCF_003348415-Contig558 | GCF_003348415_OusS6708 | 100   | 166   | 0      | 0        | 0       | 2052888 | 2053053 | 166    | 1    | 1.03E-84 | 307      | 2924072 | 166  | Human           | Unknown              | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_003716465-Contig1   | GCF_003716465_OusS6708 | 100   | 166   | 0      | 0        | 0       | 94190   | 94355   | 166    | 1    | 2.47E-86 | 307      | 95625   | 166  | Water           | Austria              | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_006802395-Contig143 | GCF_006802395_OusS6708 | 100   | 166   | 0      | 0        | 0       | 133175  | 133340  | 166    | 1    | 4.89E-86 | 307      | 134610  | 166  | Water           | Austria              | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_006802395-Contig143 | GCF_006802395_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87693   | 87858   | 1      | 166  | 1.18E-85 | 307      | 328061  | 166  | Water           | Austria              | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_009938115-Contig30  | GCF_009938115_OusS6708 | 100   | 166   | 0      | 0        | 0       | 85960   | 86125   | 1      | 166  | 1.14E-85 | 307      | 148556  | 166  | Unknown         | Unknown              | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_009938115-Contig30  | GCF_009938115_OusS6708 | 100   | 166   | 0      | 0        | 0       | 185449  | 185614  | 166    | 1    | 1.05E-84 | 307      | 2591008 | 166  | Unknown         | Hall                 | G3    | PCL | x   | x   | x     |          | x        |          |       |          |                   |                  |
| GCF_013357805-Contig1   | GCF_013357805_OusS6708 | 100   | 166   | 0      | 0        | 0       | 2482493 | 2482858 | 1      | 166  | 1.07E-84 | 307      | 3033188 | 166  | Water           | USA                  | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_013357805-Contig1   | GCF_013357805_OusS6708 | 100   | 166   | 0      | 0        | 0       | 2301321 | 2301486 | 1      | 166  | 1E-84    | 307      | 2829740 | 166  | Water           | USA                  | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_018458845-Contig372 | GCF_018458845_OusS6708 | 100   | 166   | 0      | 0        | 0       | 143998  | 144163  | 166    | 1    | 8.38E-86 | 307      | 231794  | 166  | Shrimp          | China                | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| GCF_019273635-Contig13  | GCF_019273635_OusS6708 | 100   | 166   | 0      | 0        | 0       | 3094980 | 3095145 | 0      | 166  | 1.11E-84 | 307      | 3149584 | 166  | Food            | Thailand             | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_021167035-Contig3   | GCF_021167035_OusS6708 | 100   | 166   | 0      | 0        | 0       | 144926  | 145091  | 166    | 1    | 8.4E-86  | 307      | 232853  | 166  | Homo sapiens    | Russia               | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_024745065-Contig35  | GCF_024745065_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87723   | 87888   | 1      | 166  | 1.18E-85 | 307      | 327754  | 166  | Shrimps         | India                | G9    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_025505255-Contig44  | GCF_025505255_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87519   | 88084   | 1      | 166  | 1.27E-85 | 307      | 351175  | 166  | Water           | Ghana                | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_025505355-Contig6   | GCF_025505355_OusS6708 | 100   | 166   | 0      | 0        | 0       | 283798  | 283963  | 166    | 1    | 1.09E-85 | 307      | 301281  | 166  | Environment     | Vietnam              | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_030444195-Contig68  | GCF_030444195_OusS6708 | 100   | 166   | 0      | 0        | 0       | 330464  | 330481  | 166    | 1    | 1.04E-85 | 307      | 287984  | 166  | Salt            | Russia               | G4    | x   |     |     |       |          |          |          |       |          |                   |                  |
| GCF_032819875-Contig6   | GCF_032819875_OusS6708 | 100   | 166   | 0      | 0        | 0       | 86070   | 86235   | 1      | 166  | 5.55E-86 | 307      | 152647  | 166  | Homo sapiens    | Switzerland          | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_032819875-Contig6   | GCF_032819875_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87704   | 87869   | 1      | 166  | 6.54E-86 | 307      | 181369  | 166  | Homo sapiens    | Switzerland          | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_032820295-Contig6   | GCF_032820295_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87658   | 88023   | 1      | 166  | 6.53E-86 | 307      | 180974  | 166  | Homo sapiens    | Switzerland          | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| GCF_038814095-Contig6   | GCF_038814095_OusS6708 | 100   | 166   | 0      | 0        | 0       | 16567   | 16732   | 166    | 1    | 2.35E-86 | 307      | 64275   | 166  | Shrimps         | Germany (Baltic Sea) | G3    |     |     |     |       |          | x        |          | x     |          |                   |                  |
| GCF_037101305-Contig1   | GCF_037101305_OusS6708 | 100   | 166   | 0      | 0        | 0       | 349896  | 350061  | 166    | 1    | 1.02E-84 | 307      | 2873080 | 166  | Malacambellus   | Vietnam              | G7    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_037101305-Contig1   | GCF_037101305_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87571   | 87736   | 1      | 166  | 2.71E-86 | 307      | 213793  | 166  | Homo sapiens    | Spain                | G3    | PCL |     |     |       |          |          | x        |       |          |                   |                  |
| GCF_037184335-Contig69  | GCF_037184335_OusS6708 | 100   | 166   | 0      | 0        | 0       | 82781   | 82846   | 166    | 1    | 6.52E-86 | 307      | 180831  | 166  | Shrimps         | China                | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| GCF_037462495-Contig1   | GCF_037462495_OusS6708 | 100   | 166   | 0      | 0        | 0       | 93496   | 93661   | 166    | 1    | 6.48E-86 | 307      | 179767  | 166  | water           | Germany (Baltic Sea) | G6    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_037462495-Contig1   | GCF_037462495_OusS6708 | 100   | 166   | 0      | 0        | 0       | 87638   | 88101   | 1      | 166  | 8.54E-86 | 307      | 181413  | 166  | water           | Germany (Baltic Sea) | G6    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_037462505-Contig3   | GCF_037462505_OusS6708 | 100   | 166   | 0      | 0        | 0       | 240598  | 240763  | 166    | 1    | 1.08E-85 | 307      | 299610  | 166  | water           | Germany (Baltic Sea) | G6    |     |     |     |       |          |          |          |       |          |                   |                  |
| GCF_037462505-Contig11  | GCF_037462505_OusS6708 | 100   | 166   | 0      | 0        | 0       | 66685   | 67030   | 166    | 1    | 5.02E-86 | 307      | 138514  | 166  | Sediment        | Germany (Baltic Sea) | G6    |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0050_S56-Contig1    | IRLE0050_S56_OusS6708  | 100   | 166   | 0      | 0        | 0       | 1204529 | 1204694 | 166    | 1    | 1.38E-84 | 307      | 3911265 | 166  | Sediment        | USA (Florida)        | G8    |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0073_S63-Contig9    | IRLE0073_S63_OusS6708  | 100   | 166   | 0      | 0        | 0       | 83004   | 83169   | 166    | 1    | 8.52E-86 | 307      | 180719  | 166  | Cyanobacteria   | USA (Florida)        | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0074_S64-Contig1    | IRLE0074_S64_OusS6708  | 100   | 166   | 0      | 0        | 0       | 1395502 | 1396067 | 1      | 166  | 1.43E-84 | 307      | 4034495 | 166  | Cyanobacteria   | USA (Florida)        | G8    |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0076_S65-Contig14   | IRLE0076_S65_OusS6708  | 100   | 166   | 0      | 0        | 0       | 92856   | 93021   | 166    | 1    | 4.05E-86 | 307      | 111590  | 166  | Cyanobacteria   | USA (Florida)        | G3    |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0140_S76-Contig12   | IRLE0140_S76_OusS6708  | 100   | 166   | 0      | 0        | 0       | 58886   | 59051   | 1      | 166  | 5.52E-86 | 307      | 152039  | 166  | Water           | USA (Florida)        | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0173_S76-Contig13   | IRLE0173_S76_OusS6708  | 100   | 166   | 0      | 0        | 0       | 58886   | 59051   | 1      | 166  | 5.52E-86 | 307      | 152039  | 166  | Water           | USA (Florida)        | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0187_S83-Contig1    | IRLE0187_S83_OusS6708  | 100   | 166   | 0      | 0        | 0       | 92868   | 93033   | 166    | 1    | 8.51E-86 | 307      | 180583  | 166  | Water           | USA (Florida)        | G3    | PCL |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0191_S85-Contig12   | IRLE0191_S85_OusS6708  | 100   | 166   | 0      | 0        | 0       | 92869   | 93134   | 166    | 1    | 8.52E-86 | 307      | 152039  | 166  | Water           | USA (Florida)        | G10   |     |     |     |       |          |          |          |       |          |                   |                  |
| IRLE0192_S86-Contig10   | IRLE0192_S86_OusS6708  | 100   | 166   | 0      | 0        | 0       | 87863   | 88028   | 1      | 166  | 8.52E-86 | 307      | 180891  | 166  | Water           | USA (Florida)        | G10   |     |     |     |       |          |          |          |       |          |                   |                  |

Note: The PCG and IRLE0074 clades, selected for further experiments, are highlighted.