| Primer       | Sequence                                                      | Purpose                                |
|-------------|---------------------------------------------------------------|----------------------------------------|
| Plac vanAB  |                                                              |                                        |
| 5-VanAB-UP  | tcgcatcggtacctctagaTGGAAGGTAAGGTTCCAGG                       | Upstream homology of vanAB             |
| 3-VanAB-UP  | ATTATCGGTATTATTAGTGG                                         | Upstream homology of vanAB             |
| 5-VanAB-Down| atgTACCCAAAACACC                                             | Downstream homology of vanAB           |
| 3-VanAB-Down| ccaaggctcttcagTGGGCGATGCTGGAGGGCG                           | Downstream homology of vanAB           |
| PP3735 F    | TTGTTCGATGGCTAGCAT                                         | Construction of pK19-mob-sacB-PvanA-Ptac-VanA |
| VanAB Rev   | ACGGTGCGCCCGCGGTGCG                                          | Construction of pK19-mob-sacB-PvanA-Ptac-VanA |
| VanAB-Plac-F| CTAAAAACCGATAATTCtgagTTGTGTCTGCTC                          | Construction of pK19-mob-sacB-PvanA-Ptac-VanA |
| VanAB-Plac-R| GTTTTTTGGGATAcgtgacctcctcctctgc                           | Construction of pK19-mob-sacB-PvanA-Ptac-VanA |
| gbdR        |                                                              |                                        |
| cloning F   | gacatgattacgcgaagcttAATCCAACCCAGAACCGCAC                      | Construction of pK19-mob-sacB-gbdR SNP |
| cloning R   | aaacgacgacgtaacctgGACGGTTAATACCTTACTGGAATG                    | Construction of pK19-mob-sacB-gbdR SNP |
| US V        | GTAAGTGCCCAGCTGGAGG                                         | Upstream homology of gbdR              |
| DS V        | GCCCCCTTGCGCGGAAACAA                                        | Downstream homology of gbdR            |
| Common F    | GCCTGTTACCCCTTGGACC                                        | Downstream homology of gbdR            |
| Seq R       | CTCCCTAGATGGGCGCTCCA                                     | Upstream homology of gbdR              |
| agmR        |                                                              |                                        |
| cloning F   | gacatgattacgcgaagcttCCatgTACAATAATTCTGATT                   | Construction of pK19-mob-sacB-agmR SNP |
| cloning R   | aaacgacgaccgctgaacctGCCACCTGGGACAACGACTGC                    | Construction of pK19-mob-sacB-agmR SNP |
| US V        | CAGCAAGCCAACCTGCCATT                                       | Upstream homology of agmR              |
| DS V        | CATGCCACTTTGCTGGAAG                                        | Downstream homology of agmR            |
| Common F    | TGACCCAGACCCATGACGAC                                       | Downstream homology of agmR            |
| Seq R       | GGCAGCCAGGATAGACATTG                                        | Upstream homology of agmR              |
| gstB-yadG   |                                                              |                                        |
| cloning F   | gacatgattacgcgaagcttCTGGGCTGGACTTCGAAT                      | Construction of pK19-mob-sacB-gstB SNP |
| cloning R   | aaacgacgacgctgaacctTTCAGCTCAGTGGAAGACTG                    | Construction of pK19-mob-sacB-gstB SNP |
| US V        | TCTGGAGTAGAAGCTTGCCAC                                      | Upstream homology of gstB              |
| DS V        | GATGTTGCCCCATGGTCCATGA                                      | Downstream homology of gstB            |
| Common F    | TCAAGCTGTTATGAGAAGCC                                       | Downstream homology of gstB            |
| Seq R       | GCCTGAAAGCCCATTTGCGTA                                      | Upstream homology of gstB              |
| **fleQ**                           |                                      |                                      |
|-----------------------------------|--------------------------------------|--------------------------------------|
| cloning F                         | gacatgattacgccaagcttAGATGATGCAGCAAGTGGC | Construction of pK19-mob-sacB-fleQ SNP |
| cloning R                         | aaacgacggccagttcTGTGTTGCCAGCGGTGTA    | Construction of pK19-mob-sacB-fleQ SNP |
| US V                              | GAAATGTATGACCAGGCGCG                  | Upstream homology of fleQ            |
| DS V                              | GGTGGCCTCTGCTGAT                      | Downstream homology of fleQ          |
| Common F                          | GTGAGCGGGGTGGAAGACAT                   | Downstream homology of fleQ          |
| Seq R                             | TCTGCTCGTCTTCGTCATCG                   | Upstream homology of fleQ            |

| **PP_3350**                       |                                      |                                      |
|-----------------------------------|--------------------------------------|--------------------------------------|
| 5' UP                             | gacatgattacgccaagcttTCGTTGCCGGACATCAGCAT | Construction of pK19-mob-sacB-ΔPP_3350 |
| 3' UP                             | TGTGTATTACGTCGGTGGAGAATTCCCTTCCTTTGAG  | Construction of pK19-mob-sacB-ΔPP_3350 |
| 5' DOWN                           | ACAAGTGAAGGGAAATTTGCTGCCACCAGCGATAACAA | Downstream homology of PP_3350       |
| 3' DOWN                           | aaacgacggccagttcCGATCAGCGGTGGGAT      | Upstream homology of PP_3350         |
| US V                              | CAGCAGTTGCAGCGAGGCT                   | Upstream homology of PP_3350         |
| DS V                              | CAGCAACAGCGTGCAGGCG                   | Downstream homology of PP_3350       |
| pK19-F                            | CATTAGGCACCACCGTCTTTAC                 | Sequencing pK19 plasmids            |
| pK19-R                            | CTTGCTATTACGCGCACTGC                  | Sequencing pK19 plasmids            |

| **vanA-qPCR**                     |                                      |                                      |
|-----------------------------------|--------------------------------------|--------------------------------------|
| F                                 | TCAGCGAAGACCTGGGAAATG                 | vanA qPCR                            |
| R                                 | GGCCTGATATTACGCTTC                      | vanA qPCR                            |
Table S2. ALE mutations.

| Ref. Seq | Positon | Mut. Type | Seq. Change | Gene | Details | Sy-1 | Early Iso. | Early Cult. | End Cult. | End Iso. | Early Iso. | Early Cult. | End Cult. | End Iso. | Early Iso. | Early Cult. | End Cult. | End Iso. |
|----------|---------|-----------|-------------|------|---------|------|------------|-------------|----------|----------|------------|-------------|----------|----------|------------|-------------|----------|--------|
| AE01 5451 | 195, 634 | SNP | C>T | PP_0168 | T380T (ACC>ACT) | | 0.51 | | | | | | | | | | |
| AE01 5451 | 278, 742 | INS | +A | PP_0224, sctC | intergenic (+237/+77) | | | 1 | 1 | | 1 | 1 | 1 | |
| AE01 5451 | 307, 865 | INS | +C | hslO, PP_0254 | intergenic (+880/+989) | | 1 | 1 | | 1 | 1 | | 1 | 1 |
| AE01 5451 | 336, 124 | INS | +T | PP_0278 | coding (209/237 nt) | | 1 | 1 | | 1 | 1 | | 1 | 1 |
| AE01 5451 | 358, 732 | SNP | G>A | gbdR | D67N (GAT>AAT) | | | | | | | | 1 | 1 | |
| AE01 5451 | 359, 122 | SNP | T>G | gbdR | S197A (TCC>GCC) | | 0.9 | | | | | | | 0.9 | 1 | |
| AE01 5451 | 359, 462 | SNP | A>G | gbdR | | | | | | | | | | | | | |
| AE01 5451 | 699, 722 | SNP | A>G | PP_0106, PP_23SD | intergenic (+115/+129) | | 0.53 | | | | | | | | | | |
| AE01 5451 | 747, 612 | INS | +G | PP_0638, PP_5448 | intergenic (+739/+55) | | 1 | 1 | | 1 | 1 | | 1 | 1 |
| AE01 5451 | 1,07, 0,24, 6 | INS | +G | aroP-I, PP_0928 | intergenic (+46/+96) | | | | | | | | | | | |
| AE01 5451 | 1,07, 0,24, 6 | INS | +A | aroP-I, PP_0928 | intergenic (+46/+96) | | | | | | | | | | | |
| AE01 5451 | 1,07, 0,24, 6 | INS | +GA | aroP-I, PP_0928 | intergenic (+46/+96) | | | | | | | | | | | |
| AE01 5451 | 1,07, 7,15, 3 | SNP | G>C | mreC | G178R (GGC>CGC) | | | | | | | | | 1 | 1 | |
| AE01 5451 | 545 | SNP | C>T | PP_0168 | T380T (ACC>ACT) | | | | | | | | | | | | |
| Accession | Start | End | Type  | Position | Gene  | Description | Haplotype 1 | Haplotype 2 | Haplotype 3 | Haplotype 4 | Haplotype 5 |
|-----------|-------|-----|-------|----------|-------|-------------|------------|------------|------------|------------|------------|
| AE01 5451 | 1,12  | 6,64 | INS   | +C       | tdcG-II, gcVP-I | intergenic (+99/+50) | 1 | 1 | 1 | 1 | 1 |
| AE01 5451 | 1,20  | 5,68 | SNP   | G>T      | PP_1057 | A25D (GCC>GAC) | | | | | 0.56 | 1 |
| AE01 5451 | 1,32  | 7,12 | SNP   | C>T      | PP_16SE, PP_23SE | intergenic (+97/+196) | 0.58 | 0.57 | 0.58 | 0.61 | |
| AE01 5451 | 1,41  | 9,54 | INS   | +C       | PP_1242, PP_1244 | intergenic (+37/+99) | 1 | 1 | 1 | 1 | 1 |
| AE01 5451 | 1,49  | 9,49 | SNP   | T>G      | trpS, zapE | intergenic (+74/+90) | 0.89 | | 0.88 | 0.86 | 0.93 |
| AE01 5451 | 1,49  | 9,49 | SNP   | T>G      | trpS, zapE | intergenic (+75/+89) | 0.89 | 0.91 | 0.87 | | 0.93 |
| AE01 5451 | 1,49  | 9,50 | INS   | +C       | trpS, zapE | intergenic (+83/+81) | 1 | 1 | 1 | 1 | 1 |
| AE01 5451 | 1,51  | 3,30 | SNP   | A>C      | PP_mr18, mraZ | intergenic (+235/+60) | | | | | | 1 |
| AE01 5451 | 1,93  | 2,63 | INS   | +C       | rluA, minE | intergenic (+105/+45) | 1 | 1 | 1 | 1 | 1 |
| AE01 5451 | 2,13  | 7,08 | SNP   | C>T      | fadE, gstB | intergenic (+330/+61) | | | | | | 0.79 |
| AE01 5451 | 2,13  | 7,82 | SNP   | C>T      | gstB, yadG | intergenic (+58/+76) | | | | | | 0.72 | 1 |
| AE01 5451 | 2,14  | 2,32 | SNP   | G>C      | PP_1897 | G666G (GGG>GAC) | | | | | | 0.89 | 1 |
| AE01 5451 | 3,05  | 3,66 | SNP   | A>G      | agmR | I81T (ATC>ACC) | | | | | 0.85 | 1 |
| Gene | Position | Type | Site | Feature | Description | Fold Change
|------|----------|------|------|---------|-------------|-------------
| yiaY | 3.07 | SNP | G>A | 1 | D149N (GAC>AAC) | 1 |
| yiaY | 3.07 | SNP | C>A | 1 | S272R (AGC>AGA) | 1 |
| PP_2683 | 3.07 | SNP | C>A | 1 | R314S (CGC>AGC) | 0.92 |
| PP_3394, PP_3395 | 3.84 | INS | G | 1 | +70/+28 intergenic | 1 |
| pcaJ | 4.45 | INS | A | 1 | (12/642 nt) coding | 1 |
| pcaJ | 4.45 | INS | (A)5>6 | 1 | (17/642 nt) coding | 1 |
| pcaJ | 4.45 | SNP | G>A | 1 | K6K (AAG>AAA) | 0.63 0.8 1 |
| PP_4061, PP_4063 | 4.58 | INS | G | 1 | +116/+99 intergenic | 1 1 1 1 1 1 |
| PP_4061, PP_4063 | 4.58 | INS | C | 1 | +139/+76 intergenic | 1 1 1 1 1 1 |
| PP_4234, dsbD-II | 4.80 | DEL | Δ14 bp | 1 | +284/+258 intergenic | 1 |
| PP_4234, dsbD-II | 4.80 | DEL | Δ103 bp | 0.63 1 | +286/+167 intergenic | 1 1 |
| PP_4234, dsbD-II | 4.80 | DEL | Δ14 bp | 1 | +289/+253 intergenic | 1 1 |
|   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|
| AE01 5451 | 4,93 6,03 7 | SNP | G>A | flhA | R185C (CGT>TGT) |   | 0.74 |   |   |
| AE01 5451 | 4,95 8,85 7 | SNP | G>T | fliF | G538G (GGC>GGA) |   | 0.64 |   |   |
| AE01 5451 | 4,95 9,28 8 | SNP | C>A | fliF | D395Y (GAC>TAC) |   | 0.72 |   |   |
| AE01 5451 | 4,96 4,29 4 | SNP | C>G | fleQ | R355P (CGT>CCT) |   | 1   |   |   |
| AE01 5451 | 4,96 4,66 3 | DEL | Δ1 bp | fleQ | coding (695/1476 nt) |   | 1   |   |   |
| AE01 5451 | 4,96 4,66 4 | SNP | C>A | fleQ | G232W (GGG>TGG) | 0.64 | 1   |   |   |
| AE01 5451 | 5,52 1,71 9 | SNP | C>T | lip | D23N (GAT>AAT) | 0.83 | 1   |   |   |
| AE01 5451 | 5,66 6,33 7 | SNP | G>T | nhaP | A117A (GCG>GCT) |   | 0.73 | 1   |   |
| AE01 5451 | 5,67 4,75 0 | INS | +G | rhlE-II, ycel | intergenic (+112/+28) |   | 1   | 1   | 1   |
| AE01 5451 | 5,76 2,00 1 | SNP | C>A | gpml | G351G (GGC>GGA) |   | 0.84 | 1   |   |
| AE01 5451 | 6,05 6,68 5 | SNP | G>A | oxyR | R271H (CGC>CAC) |   | 0.7  |   | 1   |
Table S3. Converged mutations.

| Ref Seq | Position | Mutation Type | Sequence Change | Gene   | Details                  | Details                  | Culture 1 | Culture 2 | Culture 3 |
|---------|----------|---------------|-----------------|--------|--------------------------|--------------------------|-----------|-----------|-----------|
| AE0154  | 4,964,29 | SNP           | C>G             | fleQ   | R355P (CGT>CCT)          |                          | 1         |           |           |
| 51      | 4        |               |                 |        |                          |                          |           |           |           |
| AE0154  | 4,964,66 | DEL           | Δ1 bp           | fleQ   | coding (695/1476 nt)     |                          |           |           | 1         |
| 51      | 3        |               |                 |        |                          |                          |           |           |           |
| AE0154  | 4,964,66 | SNP           | C>A             | fleQ   | G232W (GGG>TGG)          |                          |           |           | 1         |
| 51      | 4        |               |                 |        |                          |                          |           |           |           |
| AE0154  | 359,122  | SNP           | T>G             | gbdR   | S197A (TCC>GCC)          |                          | 1         |           |           |
| 51      |          |               |                 |        |                          |                          |           |           |           |
| AE0154  | 358,732  | SNP           | G>A             | gbdR   | D67N (GAT>AAT)           |                          |           | 1         |           |
| 51      |          |               |                 |        |                          |                          |           |           |           |
| AE0154  | 359,462  | SNP           | A>G             | gbdR   | K310R (AAG>AGG)          |                          |           |           | 1         |
| 51      |          |               |                 |        |                          |                          |           |           |           |
| AE0154  | 2,137,82 | SNP           | C>T             | gstB,  | intergenic (+58/+76)     |                          | 1         |           |           |
| 51      | 2        |               |                 | yadG   |                          |                          |           |           |           |
| AE0154  | 3,071,59 | SNP           | G>A             | yiaY   | D149N (GAC>AAC)          |                          |           |           | 1         |
| 51      | 2        |               |                 |        |                          |                          |           |           |           |
| AE0154  | 3,071,96 | SNP           | C>A             | yiaY   | S272R (AGC>AGA)          |                          |           |           |           |
| 51      | 3        |               |                 |        |                          |                          |           |           |           |
Figure S1. qPCR analysis of *gllA* and *gllB* expression. The expression levels were normalized to *gllA* or *gllB* expression in wild-type *P. putida* KT2440 grown in glucose.