Supplementary Information

Widely used benzalkonium chloride disinfectants can promote antibiotic resistance

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Supporting results and discussion

Integrative and conjugative elements (ICEs)

In contrast to plasmids, ICEs can be integrated into the host chromosome via phage integrase-mediated chromosomal integration at specific sites encoding tRNA genes (1). In GI-2, we found a tyrosine-based site-specific recombinase CMGI-2, and a tRNA^{Gly}, which was located 80 bp upstream of GI-2 and overlapped, over 22bp, with a sequence that was duplicated at the other end of GI-2 (Supplementary Fig. 3). These results suggested that tyrosine-based recombinase might have catalyzed the site-specific integration of GI-2 in *P. aeruginosa* DPB. However, GI-2 also harbored a transposase DDE domain protein, which is known to employ various mechanisms of recombination with promiscuous integration sites (2), indicating that non-site-specific integration is also possible for this ICE. Furthermore, the presence of plasmid replication initiator (*repA*) in GI-2 suggested that GI-2 may be also prone to plasmid-like replication, similar to several other known ICEs (3), which blur the line dividing ICEs and conjugative plasmids.

BAC-adaptation in *P. aeruginosa*

In contrast to all *P. aeruginosa* DP BAC(+) populations, *P. aeruginosa* DPB BAC(+) and BAC(-) populations did not showed higher MIC to the membrane-active antibiotic polymyxin B except for DPB BAC(+)_1 compared to the ancestor even thought they all had mutations in *pmrB*, albeit at different locations than DP BAC(+) (Table 2 and Supplementary Table 3B). The mutations to *pmrB* in DPB BAC(+) and BAC(-) populations apparently did not affect their polymyxin B resistance level relative to their ancestor (but only their BAC tolerance level) because the level of polymyxin resistance depends on the type of mutation in *pmrB* (e.g., exact location and combinations of mutation) and possibility of presence of other determinants for resistance phenotype (4). For instance, repeated passages without polymyxin in the growth media results in loss of resistance in some cases, suggesting that the *pmrAB* locus is not the only determinant of the resistance phenotype (4). These other (unknown) determinants and their epistatic effects on the mutation on the *pmrB* gene presumably accounted for the lack of increased polymyxin B resistance in DPB BAC(+) populations. Consistent with these interpretations, we found that the expression level of *arnBCADTEF* operon under BAC-free (but not under BAC-exposed) conditions in all DPB BAC(+) populations was not significantly different from that of their ancestor, and almost no expression of *arnE* and *arnF* (Supplementary Table 6A). However, when BAC was added to the growth media before taking the sample for transcriptomics, overexpression of *arnBCADT* and almost no expression of *arnE* and *arnF* were observed (Supplementary Table 6B). *arnE* and *arnF* are presumably responsible for transportation of undecaprenyl phosphate-α-L-Ara4N from inner membrane to outer surface (5), which is required for the last step of the synthesis of L-Ara4N modified lipidA. Therefore, it appears that, at least in *P. aeruginosa* DPB, overexpression of *arnBCAD* produces undecaprenyl phosphate-α-L-Ara4N in the inner membrane, which is important for increased BAC tolerance but not relevant for conferring polymyxin B resistance; the latter apparently requires the transportation of the undecaprenyl phosphate-α-L-Ara4N to the outer membrane. And, incomplete expression of *arnBCADTEF* operon, especially *arnE* and *arnF*, presumably accounted for the fact that DPB BAC(+) populations did not show increased polymyxin B resistance under BAC exposed condition compared to their ancestor (Supplementary Table 3C). Consistent with these findings and interpretations, the

DPB BAC(-)_1 population, which showed decreased BAC tolerance relative to the other two *P. aeurginosa* DPB BAC(-) replicate populations, had 20bp insertion mutation in *pmrA*, and consequently the expression level of *arnBCADTEF* operon was not different from that of ancestor or control under the BAC-exposed condition (Supplementary Table 4D and 6C).

All *P. aeruginosa* DP BAC(-) populations showed loss of polymyxin B resistance relative to DP BAC(+) populations even though they had fixed mutations in *pmrB* (Figure 3A and Table 2). Read alignment of DP BAC(-) datasets against assembled contigs of *P. aeruginosa* DP ancestor suggested that subpopulations of *P. aeruginosa* DP BAC(-)_2 and DP BAC(-)_3 had different mutations in *pmrB* and *pmrA* compared to those in *P. aeruginosa* DP BAC(+) populations (Supplementary Table 5). This finding may explain the loss of polymyxin B resistance in these populations since the location and combination of mutations in *pmrB* are known to affect the level of polymyxin B resistance (discussed above). In the case of population *P. aeruginosa* DP BAC(-)_1, which did not have the mutations in *pmrB* or *pmrA* that P. aeruginosa DP BAC(-)_2 and BAC(-)_3 had, it is likely that mutations elsewhere in the genome during the BAC(-) phase were responsible for loss of polymyxin B resistance. For instance, this population uniquely had a 15bp insertion mutation in the quorum sensing regulator gene *lasR* (Supplementary Table 4C). Expression of more than 300 genes, including MFS transporters, RND efflux pump genes, and two-component response regulators, are regulated by two acyl-homoserine lactone systems, LasR-LasI and RhlR-RhlI (6, 7). Therefore, it is likely that the mutation in *lasR* might have induced expression changes in the genes related to polymyxin B resistance in *P. aeruginosa* BAC(-)_1 population, although this hypothesis awaits experimental validation. In summary, BAC exposure selected for the mutations in *pmrB* that conferred increased BAC-tolerance in all *P. aeruginosa* populations, but only specific mutations conferred increased polymyxin resistance in the DP (but not the DPB) genetic background. For the remaining of mutations, gene expression data provided testable hypotheses for explaining the lack of increased polymyxin B resistance.

References

1. **Grindley ND, Whiteson KL, Rice PA.** 2006. Mechanisms of site-specific recombination. Annu. Rev. Biochem. **75:**567-605.
2. **Hickman AB, Chandler M, Dyda F.** 2010. Integrating prokaryotes and eukaryotes: DNA transposases in light of structure. Critical reviews in biochemistry and molecular biology **45:**50-69.
3. **Lee CA, Babic A, Grossman AD.** 2010. Autonomous plasmid ‐ like replication of a conjugative transposon. Molecular microbiology **75:**268-279.
4. **Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP.** 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrobial agents and chemotherapy **56:**1019-1030.
5. **Yan A, Guan Z, Raetz CR.** 2007. An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. Journal of Biological Chemistry **282:**36077-36089.
6. **Schuster M, Lostroh CP, Ogi T, Greenberg E.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. Journal of bacteriology **185:**2066-2079.
7. **Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. Journal of bacteriology **185:**2080-2095.

Supplementary Figures and Tables

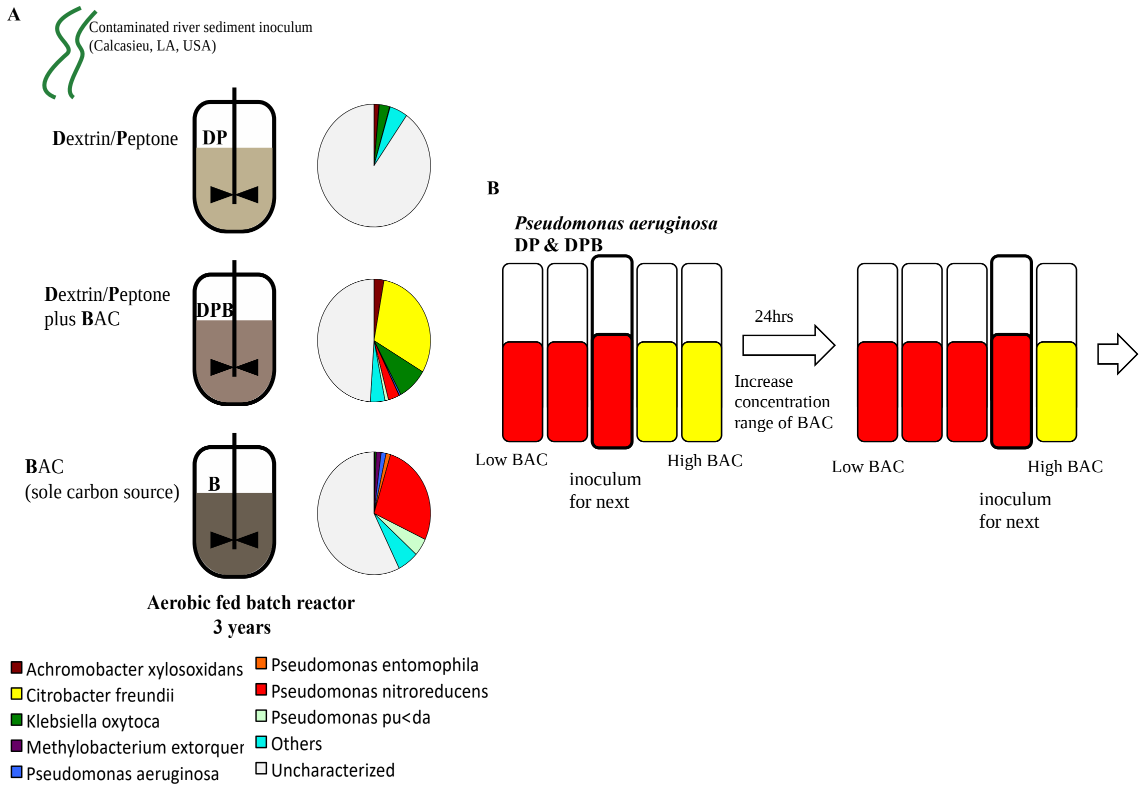
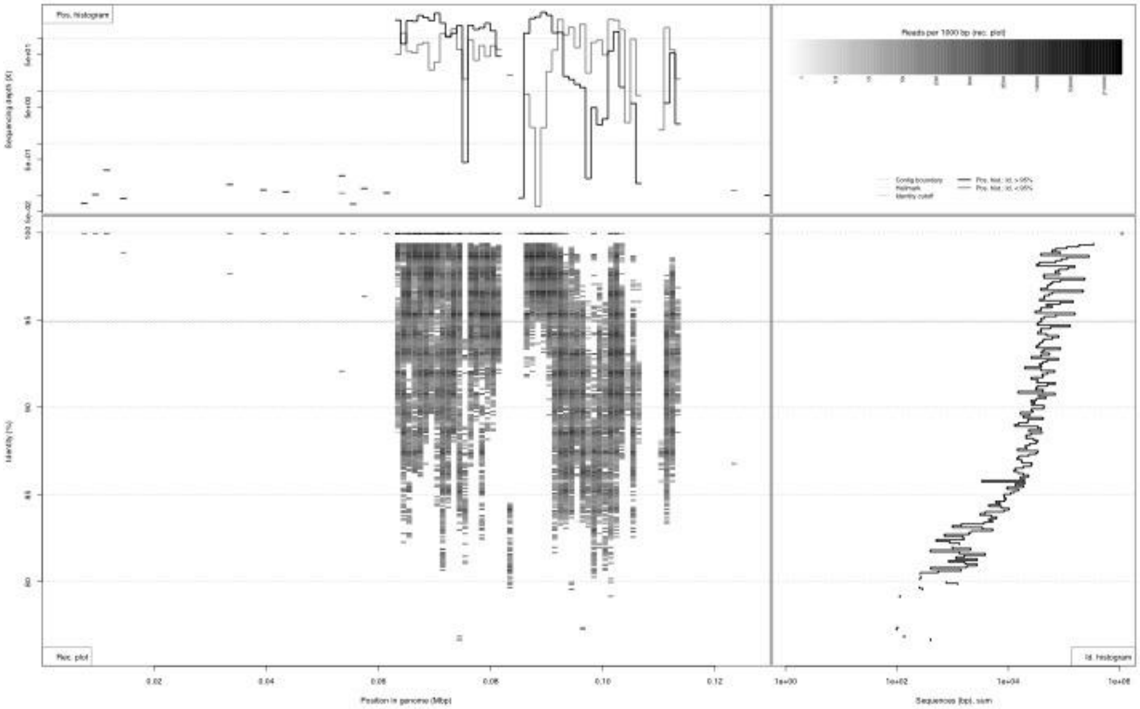


Figure S1. | Bioreactor development and adaptive evolution experiment. (A) Bioreactors were built with a microbial community inoculum originating from a river sediment (Calcasieu River, USA). The substrates and community ages for each bioreactor are shown. Colors represent the abundant community members, making >1% of the total (see figure key). (B) Schematic representation of the adaptive evolution experimental design. 1% aliquot from the culture that showed a ratio higher than 0.2 of relative growth compared to the control (measured by optical density; red test tubes) for the highest concentration of BAC tested was used as inoculum for the next round with higher BAC concentrations. Yellow tubes denote no growth (due to toxicity by the BAC concentration).

A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5e-01
5e+00
5e+01
Reads per 1000 bp (rec. plot)
Contig boundary
Hallmark
Identity cutoff
Pos. hist.: Id. > 95%
Pos. hist.: Id. < 95%
Identity (%)
100
95
90
85
80
Rec. plot
0.02
0.04
0.06
0.08
0.10
0.12
Position in genome (Mbp)
Id. histogram
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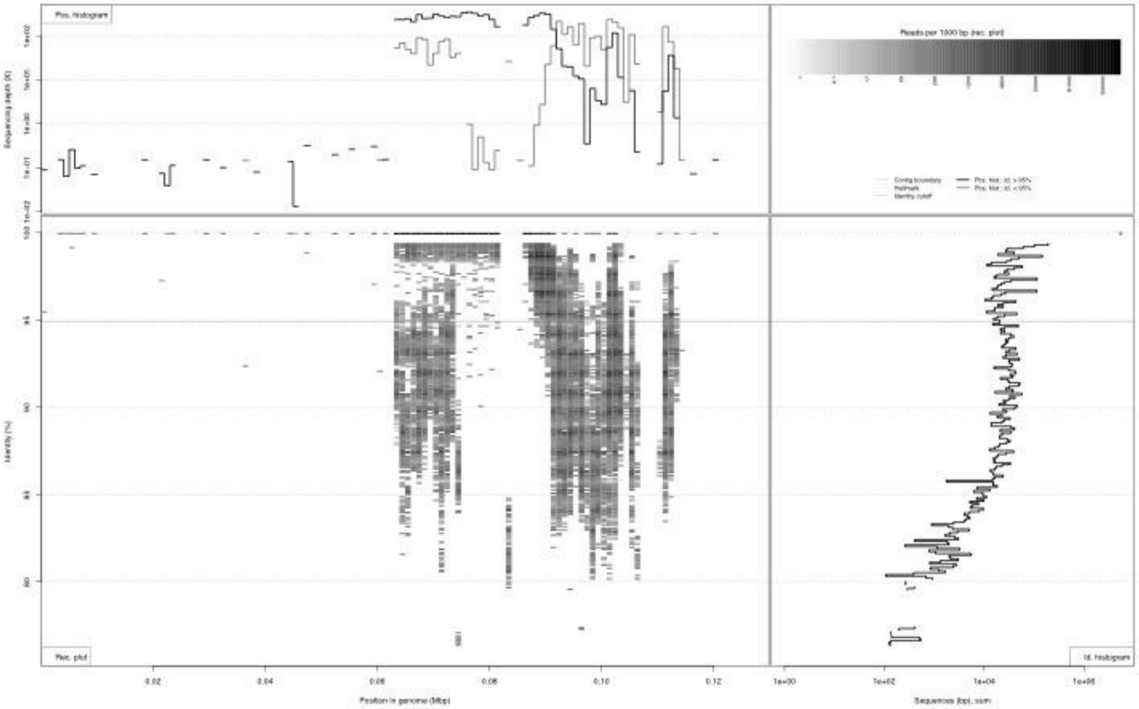
1e+04
1e+06
Sequences (bp), sum

B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Contig boundary
Hallmark
Identity cutoff
Pos. hist.: Id. > 95%
Pos. hist.: Id. < 95%
Identity (%)
100
95
90
85
80
Rec. plot
0.02
0.04
0.06
0.08
0.10
0.12
Position in genome (Mbp)
Id. histogram
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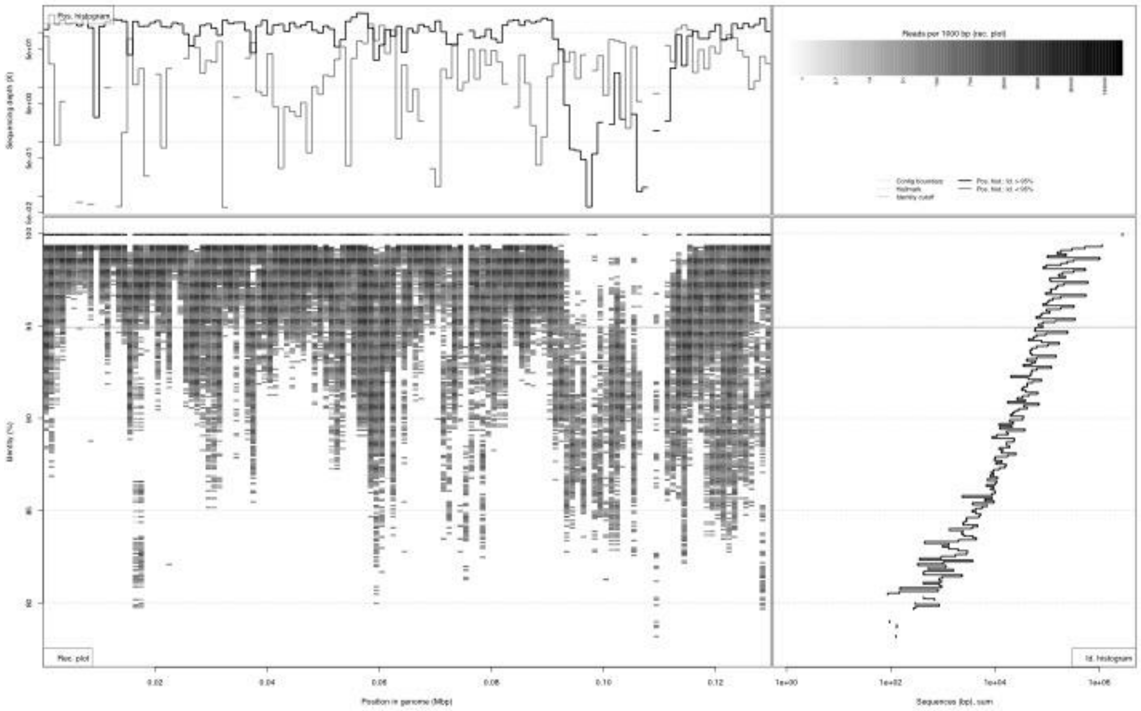
1e+02
1e+04
1e+06
Sequences (bp), sum

C


Pos. histogram
Sequencing depth (X)
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Reads per 1000 bp (rec. plot)
Contig boundary
Hallmark
Identity cutoff
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100
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Rec. plot
0.02
0.04
0.06
0.08
0.10
0.12
Position in genome (Mbp)
Id. histogram
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1e+02
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1e+06
Sequences (bp), sum

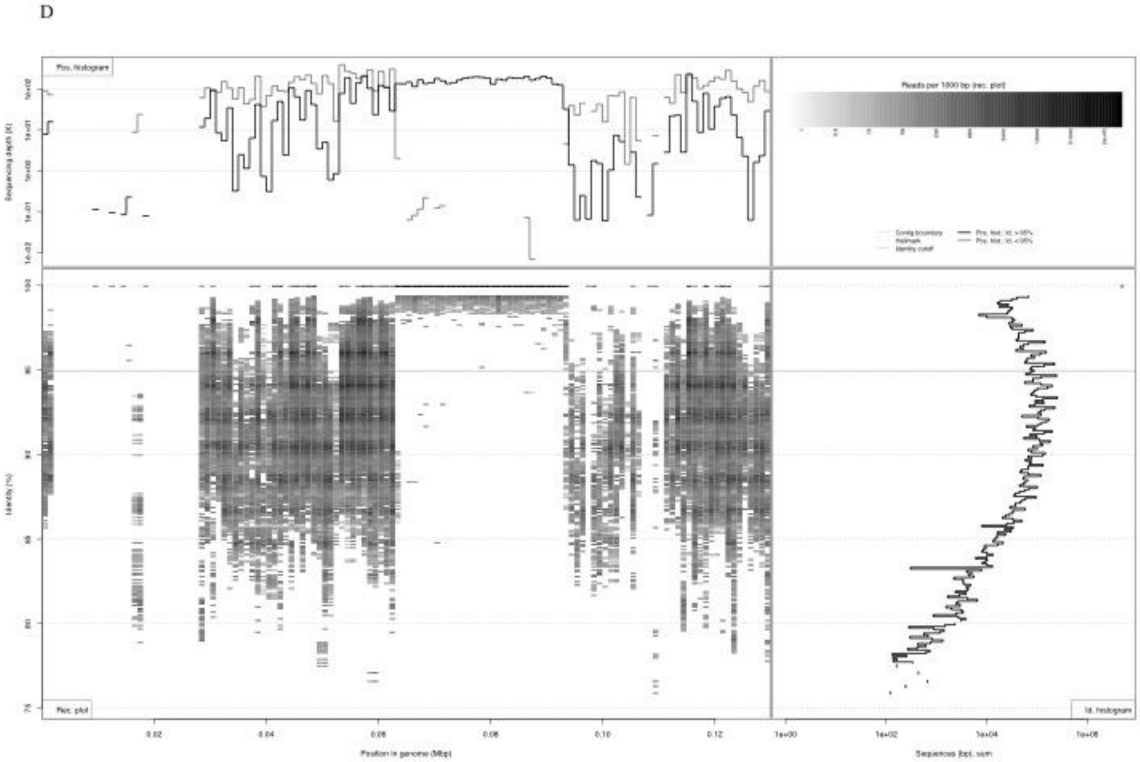

Figure S2. | Fragment recruitment plots of reads of isolate genomes against the *P. aeruginosa* strain DPB GI-2 reference sequence. The fragment recruitment plot consists of four panels: (1) Bottom left panel represents the individual reads recruited against GI-2, placed by location (x-axis) and percent of identity (y-axis) to GI-2 sequence; (2) Top left panel represents sequencing depth across the reference GI-2 sequence, i.e., number of times each nucleotide base of the reference is covered by reads, in logarithmic scale; (3) Bottom right panel represents identity histogram of mapping reads, i.e., how many bases are found at each value of nucleotide identity, in logarithmic scale; and (4) Top right panel represents color scale for the number of stacked reads in bottom left panel. Panels represent reads from: (A) *K. michiganensis* DPB, (B) *Citrobacter freundi* DPB, (C) *Achromobacter sp.* DPB, and (D) *Achromobacter sp.* B.

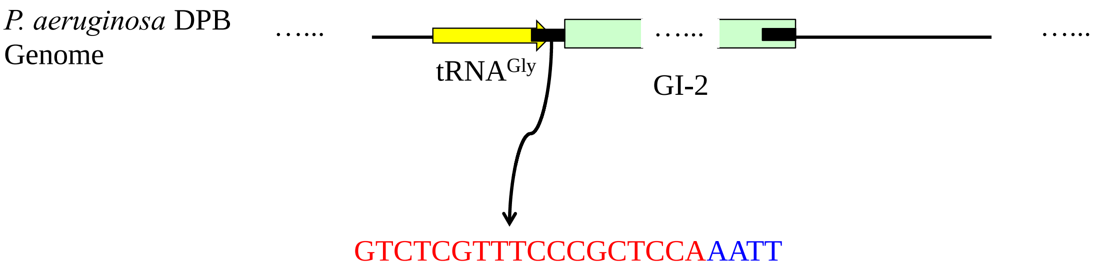

Figure S3. | Schematic representation of the region where the GI-2 element was integrated in the *P. aeruginosa* DPB genome. The yellow arrow indicates the sequence of the tRNA^{Gly} gene. The black rectangles indicate identical short repeated sequences (22 bp) that most likely belong to the *attB* integration site i.e., red-colored nucleotide bases are 100% identical to the tRNA gene while the blue ones do not match the tRNA gene sequence.

Table S1. Gene content of GI-2. Gray shading highlights the genes encoding defining features of integrative and conjugative elements (ICEs). Gene sequences can be found at http://enve-omics.ce.gatech.edu/data/bac

Gene ID	Bioinformatics-based Functional Annotation
1_2121	MULTISPECIES: integrase [Proteobacteria]
1_2122	MULTISPECIES: dye-decolorizing peroxidase (DyP),encapsulated subgroup [Proteobacteria]
1_2123	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2124	MULTISPECIES: LysR family transcriptional regulator [Proteobacteria]
1_2125	MULTISPECIES: formaldehyde dehydrogenase glutathione-independent [Proteobacteria]
1_2126	PREDICTED: protocadherin-12 [Jaculus jaculus]
1_2127	LysR family transcriptional regulator [Pseudomonas aeruginosa]
1_2128	Transposase DDE domain protein (TnpA) [Pseudomonas aeruginosa]
1_2129	MULTISPECIES: excinuclease ABC subunit UvrB [Proteobacteria]
1_2130	MULTISPECIES: LysR family transcriptional regulator [Proteobacteria]
1_2131	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2132	MULTISPECIES: GNAT family acetyltransferase [Proteobacteria]
1_2133	MULTISPECIES: nitrilase [Proteobacteria]
1_2134	MULTISPECIES: S-(hydroxymethyl)glutathione dehydrogenase/class III alcohol dehydrogenase [Proteobacteria]
1_2135	MULTISPECIES: VOC family protein [Proteobacteria]
1_2136	MULTISPECIES: DUF3422 domain-containing protein [Proteobacteria]
1_2137	MULTISPECIES: S-formylglutathione hydrolase [Proteobacteria]
1_2138	MULTISPECIES: NAD(P)/FAD-dependent oxidoreductase [Proteobacteria]
1_2139	MULTISPECIES: replication-associated recombination protein A [Proteobacteria]
1_2140	MULTISPECIES: Na+/H+ antiporter NhaA [Proteobacteria]
1_2141	MULTISPECIES: excinuclease ABC subunit UvrA [Proteobacteria]
1_2142	MULTISPECIES: relaxase [Proteobacteria]
1_2143	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2144	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2145	MULTISPECIES: DUF3742 domain-containing protein [Proteobacteria]
1_2146	MULTISPECIES: conjugal transfer protein TraG [Proteobacteria]
1_2147	MULTISPECIES: hypothetical protein [Pseudomonas]

1_2148	MULTISPECIES: integrating conjugative element protein [Gammaproteobacteria]
1_2149	MULTISPECIES: TIGR03756 family integrating conjugative element protein [Proteobacteria]
1_2150	MULTISPECIES: TIGR03757 family integrating conjugative element protein [Proteobacteria]
1_2151	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2152	MULTISPECIES: DSBA oxidoreductase [Proteobacteria]
1_2153	MULTISPECIES: conjugative transfer ATPase [Proteobacteria]
1_2154	MULTISPECIES: TIGR03751 family conjugal transfer lipoprotein [Proteobacteria]
1_2155	MULTISPECIES: TIGR03752 family integrating conjugative element protein [Proteobacteria]
1_2156	MULTISPECIES: TIGR03749 family integrating conjugative element protein [Proteobacteria]
1_2157	MULTISPECIES: TIGR03746 family integrating conjugative element protein [Proteobacteria]
1_2158	MULTISPECIES: TIGR03750 family conjugal transfer protein [Proteobacteria]
1_2159	MULTISPECIES: TIGR03745 family integrating conjugative element membrane protein [Proteobacteria]
1_2160	MULTISPECIES: TIGR03758 family integrating conjugative element protein [Proteobacteria]
1_2161	MULTISPECIES: plasmid Ignore, RAQPRD family [Proteobacteria]
1_2162	MULTISPECIES: TIGR03747 family integrating conjugative element membrane protein [Proteobacteria]
1_2163	MULTISPECIES: conjugative coupling factor TraD, PFGI-1 class [Proteobacteria]
1_2164	MULTISPECIES: integrating conjugative element protein [Proteobacteria]
1_2165	MULTISPECIES: lytic transglycosylase [Proteobacteria]
1_2166	MULTISPECIES: TIGR03759 family integrating conjugative element protein [Proteobacteria]
1_2167	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2168	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2169	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2170	MULTISPECIES: DEAD/DEAH box helicase [Gammaproteobacteria]
1_2171	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2172	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2173	MULTISPECIES: SAM-dependent methyltransferase [Proteobacteria]
1_2174	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2175	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2176	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2177	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2178	MULTISPECIES: DUF3275 domain-containing protein [Proteobacteria]

1_2179	MULTISPECIES: DUF3577 domain-containing protein [Proteobacteria]
1_2180	MULTISPECIES: DUF3085 domain-containing protein [Proteobacteria]
1_2181	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2182	MULTISPECIES: DUF2274 domain-containing protein [Proteobacteria]
1_2183	MULTISPECIES: conjugative transfer protein TrbI [Proteobacteria]
1_2184	MULTISPECIES: P-type conjugative transfer protein TrbG [Proteobacteria]
1_2185	MULTISPECIES: conjugal transfer protein TrbF [Proteobacteria]
1_2186	MULTISPECIES: P-type conjugative transfer protein TrbL [Proteobacteria]
1_2187	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2188	MULTISPECIES: P-type conjugative transfer protein TrbJ [Proteobacteria]
1_2189	MULTISPECIES: conjugal transfer protein TrbE [Proteobacteria]
1_2190	MULTISPECIES: conjugal transfer protein TrbD [Proteobacteria]
1_2191	MULTISPECIES: conjugal transfer protein TrbC [Proteobacteria]
1_2192	MULTISPECIES: P-type conjugative transfer ATPase TrbB [Proteobacteria]
1_2193	MULTISPECIES: ribbon-helix-helix protein, CopG family [Proteobacteria]
1_2194	MULTISPECIES: conjugal transfer protein TraG [Proteobacteria]
1_2195	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2196	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2197	hypothetical protein [Pseudomonas aeruginosa]
1_2198	MULTISPECIES: QacE family quaternary ammonium compound efflux SMR transporter [Proteobacteria]
1_2199	MULTISPECIES: LysR family transcriptional regulator [Proteobacteria]
1_2200	TolC family protein [Pseudomonas sp. NBRC 111127]
1_2201	MULTISPECIES: TetR/AcrR family transcriptional regulator [Proteobacteria]
1_2202	MULTISPECIES: efflux RND transporter periplasmic adaptor subunit [Proteobacteria]
1_2203	MULTISPECIES: AcrB/AcrD/AcrF family protein [Proteobacteria]
1_2204	MULTISPECIES: DUF3313 domain-containing protein [Proteobacteria]
1_2205	MULTISPECIES: ABC transporter permease [Proteobacteria]
1_2206	MULTISPECIES: ABC transporter ATP-binding protein [Proteobacteria]
1_2207	MULTISPECIES: MCE family protein [Proteobacteria]
1_2208	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2209	MULTISPECIES: DUF3363 domain-containing protein [Proteobacteria]
1_2210	MULTISPECIES: peptidase [Proteobacteria]
1_2211	MULTISPECIES: DUF2840 domain-containing protein [Proteobacteria]
1_2212	chromosome partitioning protein ParB [Pseudomonas aeruginosa]
1_2213	MULTISPECIES: plasmid partitioning protein [Proteobacteria] or Chromosome (plasmid) partitioning protein ParA [Achromobacter xylosoxidans]
1_2214	MULTISPECIES: replication initiator and transcriptional regulator protein [Proteobacteria] or RepA replication protein [Pseudomonas aeruginosa]
1_2215	MULTISPECIES: DNA-binding protein [Proteobacteria]

1_2216	MULTISPECIES: DUF2285 domain-containing protein [Proteobacteria]
1_2217	MULTISPECIES: DUF2958 domain-containing protein [Bacteria]
1_2218	MULTISPECIES: XRE family transcriptional regulator [Proteobacteria]
1_2219	MULTISPECIES: DUF736 domain-containing protein [Proteobacteria]
1_2220	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2221	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2222	MULTISPECIES: DUF945 domain-containing protein [Proteobacteria]
1_2223	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2224	MULTISPECIES: DUF2958 domain-containing protein [Proteobacteria]
1_2225	MULTISPECIES: RadC-like protein [Proteobacteria]
1_2226	MULTISPECIES: ArsR family transcriptional regulator [Proteobacteria]
1_2227	MULTISPECIES: arsenate reductase ArsC [Proteobacteria]
1_2228	MULTISPECIES: arsenical-resistance protein [Proteobacteria]
1_2229	MULTISPECIES: arsenate reductase (glutaredoxin) [Proteobacteria]
1_2230	MULTISPECIES: arsenical resistance protein ArsH [Proteobacteria]
1_2231	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2232	MULTISPECIES: DUF1016 domain-containing protein [Proteobacteria]<>PREDICTED: uncharacterized protein LOC105828055 [Monomorium pharaonis]
1_2233	site-specific recombinase, phage integrase family [Bordetella bronchiseptica GA96-01] or tyrosine-based site-specific recombinase CMGI-2 [Pseudomonas sp. ATCC 13867]
1_2234	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2235	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2236	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2237	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2238	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2239	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2240	MULTISPECIES: DNA cytosine methyltransferase [Proteobacteria]
1_2241	MULTISPECIES: DNA topoisomerase III [Proteobacteria]
1_2242	MULTISPECIES: single-stranded DNA-binding protein [Proteobacteria]
1_2243	MULTISPECIES: DUF3158 domain-containing protein [Proteobacteria]
1_2244	MULTISPECIES: TIGR03761 family integrating conjugative element protein [Proteobacteria]
1_2245	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2246	MULTISPECIES: DUF2857 domain-containing protein [Proteobacteria]
1_2247	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2248	MULTISPECIES: hypothetical protein [Proteobacteria]
1_2249	MULTISPECIES: ParA family protein [Proteobacteria]
1_2250	MULTISPECIES: AlpA family phage regulatory protein [Proteobacteria]
1_2251	MULTISPECIES: hypothetical protein [Proteobacteria]

Table S2. MIC values of transformants carrying efflux pump genes

Strain	MIC, mg/L		
	BAC	Tetracycline	Rifampin
PA0509-PBBRMCS4	12.5	0.2	12.5
PA0509/pBBRsugE-A*	25	0.2	12.5
PA0509/pBBRsugE-B	12.5	0.2	12.5
PA0509/pBBRABC	12.5	0.2	25

***** PA0509/pBBRsugE-A showed 89.65% reduction in growth at 12.5 mg of BAC/L only once out of three independent replicates. The other two replicates showed ≤ 80% growth reduction at 12.5 mg of BAC/L and ≥ 80% reduction in growth at 25 mg of BAC/L. Hence, 25 mg/L is reported in the Table for BAC.

Table S3. | MIC of antibiotics for *P. aeruginosa* DP & DPB evolved populations. (A) MIC of antibiotics in *P.aeruginosa* DP. (B) MIC of antibiotics in *P.aeruginosa* DPB. (C) MIC of antibiotics supplemented with BAC in *P.aeruginosa* DPB.

A

Populations	MIC, mg/L					
	Tetracycline	Ciprofloxacin	Chloramphenicol	Kanamycin	Rifampin	Ampicillin
P. aeruginosa DP BAC(+)_1	12.5	0.4	25	> 400	25	3200
P. aeruginosa DP BAC(+)_2	12.5	< 0.1	25	50	6.25	400
P. aeruginosa DP BAC(+)_3	12.5	< 0.1	50	100	25	1600
P. aeruginosa DP Control_1	6.25	< 0.1	50	100	25	1600
P. aeruginosa DP BAC(-)_1	6.25	0.025	< 12.5	400	6.25	6400
P. aeruginosa DP BAC(-)_2	3.2	0.1	25	100	< 3.2	100
P. aeruginosa DP BAC(-)_3	12.5	0.8	200	200	12.5	3200
P. aeruginosa DP ancestor	12.5	0.1	50	100	12.5	3200
P. aeruginosa DP Control_2	6.25	0.1	50	100	12.5	1600

B

populations	MIC, mg/L						
	Tetracycline	Ciprofloxacin	Chloramphenicol	Polymyxin B	Kanamycin	Rifampin	Ampicillin
P. aeruginosa DPB BAC(+)_1	12.5	0.2	50	0.8	200	25	12800
P. aeruginosa DPB BAC(+)_2	3.125	0.2	25	0.2	50	6.25	400
P. aeruginosa DPB BAC(+)_3	12.5	0.2	50	0.4	100	25	3200
P. aeruginosa DPB BAC(-)_1	6.25	< 0.1	50	0.4	200	25	6400
P. aeruginosa DPB BAC(-)_2	6.25	0.1	12.5	0.4	50	12.5	< 200
P. aeruginosa DPB BAC(-)_3	12.5	0.2	50	0.4	100	25	1600
P. aeruginosa DPB ancestor	6.25	0.2	100	0.2	100	25	3200
P. aeruginosa DPB Control_1	12.5	0.4	100	0.4	100	25	3200
P. aeruginosa DPB Control_2	6.25	0.2	50	0.4	100	12.5	3200

C

populations (LB+BAC cultured)	MIC, mg/L				
	Chloramphenicol +BAC(100mg/L)	Polymyxin B+BAC(100mg/L)	Kanamycin+BAC(100 mg/L)	Rifampin+BAC(100 mg/L)	Ampicillin+BAC(100 mg/L)
P. aeruginosa DPB BAC(+)_1	100	0.8	200	6.25	6400
P. aeruginosa DPB BAC(+)_2	25	0.4	50	6.25	< 200
P. aeruginosa DPB BAC(+)_3	25	0.4	50	< 3.2	3200
P. aeruginosa DPB ancestor	50	0.4	200	< 3.2	6400
P. aeruginosa DPB Control_1	50	0.2	200	< 3.2	6400

Table S4A. Details of SNP mutations *in P. aeruginosa* DP evolved populations. Mutations observed in control populations were removed to consider long-term exposure of LB media effect. Gray shading indicates populations with a mutation.

Contig	**Annotation**	**Gene position (Contig)**	**Mutation in contig**	**Amino acid change**	**BAC(+)_1**	**BAC(+)_2**	**BAC(+)_3**	**BAC(-)_1**	**BAC(-)_2**	**BAC(-)_3**
Cont130	tolQ-type transporter	10,800	C→A	Glu → Stop			■			■

Cont130	ribonuclease E	5,273	A→G	Silent		■		■	■	
Cont147	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexD	66,759	T→C	Asn → Ser		■			■	
Cont147	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexD	65,685	A→C	Val → Gly			■			■
Cont174	Type III secretion outer membrane protein PopN precursor	100,586	C→T	Silent						■
Cont246	hypothetical protein H123_32466, partial	25	A→G	Silent	■					
Cont41	hemagglutination protein, partial	52,142	G→A	Silent			■			
Cont53	PmrB: two-component regulator system signal sensor kinase PmrB	8,288	A→G	Phe → Leu	■	■		■	■	
Cont53	PmrB: two-component regulator system signal sensor kinase PmrB	8,636	T→C	Met → Val			■			■
Cont94	aluminum activated malate transporter family protein	33,856	G→A	Thr → Ile						■

Cont188	Full=UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase; AltName: Full=Protein EnvA; AltName: Full=UDP-3-O-acyl-GlcNAc deacetylase	80,476	A→T	Leu → Gln		■			■	
Cont188	Full=RNA polymerase sigma-54 factor	134,734	T→A	Trp → Arg						■
Cont240	hypothetical protein of bacteriophage Pf1	739	C→T	Silent			■	■	■	
Cont245	glycoprotein, partial	1,247	C→T	Ala → Thr			■			
Cont224	glycoprotein, partial	1,031	G→A	Silent	■					
Cont55	hypothetical protein PA0943	63,040	G→C	Tyr → Stop	■			■		
Cont55	lipopolysaccharide biosynthetic protein LpxO2	58,466	G→C	Arg → Gly			■			■
Cont99	Full=DNA-directed RNA polymerase subunit alpha; Short=RNAP subunit alpha; AltName: Full=RNA polymerase subunit alpha; AltName: Full=Transcriptase subunit alpha	13,252	C→G	Leu → Val	■			■		

Cont195	transposase insM for insertion sequence element IS600	625	G→A	Silent		■			■	
Cont195	transposase insM for insertion sequence element IS600	1,180	A→G	Silent		■				
Cont195	transposase insM for insertion sequence element IS600	903	A→G	Lys → Arg					■	
Cont210	alpha-1,6-rhamnosyltransferase	12,406	A→C	His → Pro		■			■	
Cont44	hypothetical protein PA3904	10,230	G→A	Silent		■			■	
Cont85	RecName: Full=Chaperone SurA; AltName: Full=Peptidyl-prolyl cis-trans isomerase SurA; Short=PPIase SurA; AltName: Full=Rotamase SurA; Flags: Precursor	67,351	T→G	Thr → Pro		■	■		■	■
	RecName: Full=Chaperone SurA; AltName: Full=Peptidyl-prolyl cis-trans isomerase SurA; Short=PPIase SurA; AltName: Full=Rotamase SurA; Flags: Precursor	21,278	C→T	Silent						■

Cont142	lipopolysaccharide biosynthetic protein LpxO1	12,214	A→G	His → Arg			■			■
Cont165	peptidase S41	67,419	G→A	Gly → Ser			■			■
Cont56	type IV secretion protein Rhs	1,398	T→C	Silent				■		
Cont84	RecName: Full=Denitrification regulatory protein NirQ	3,964	G→A	Silent						■

Table S4B. Details of SNP mutations in *P. aeruginosa* DPB evolved populations. Mutations observed in control populations were removed to consider long-term exposure of LB media effect. Gray shading indicates populations with a mutation.

Contig	**Annotation**	**Gene position (Contig)**	**Mutation in contig**	**Amino acid change**	**BAC(+)_1**	**BAC(+)_2**	**BAC(+)_3**	**BAC(-)_1**	**BAC(-)_2**	**BAC(-)_3**
Cont110	two-component response regulator CbrB	38,368	T→C	Ile→Thr	■					
Cont110	PmrB: two-component regulator system signal sensor kinase PmrB	94,923	G→A	Val→Ile		■			■	
Cont110	PmrB: two-component regulator system signal sensor kinase PmrB	95,407	T→C	Leu→Pro	■			■		
Cont110	PmrB: two-component regulator system signal sensor kinase PmrB	95,266	G→C	Gly→Ala			■			■
Cont64	glycoprotein, partial	21,505	C→G	Silent			■			

Cont67	cbb3-type cytochrome c oxidase subunit 1	149,131	C→G	Silent					■	
Cont67	cbb3-type cytochrome c oxidase subunit 1	149,266	C→G	Silent					■	
Cont77	serine protease MucD	61,376	A→G	Val→Ala		■			■	
Cont98	hemagglutination protein, partial	52,144	G→A	Silent			■			
Cont98	hemagglutination protein, partial	52,205	C→A	Pro→Lys			■			■
Cont98	hemagglutination protein, partial	52,206	C→A				■			■
Cont104	RecName: Full=Multidrug efflux pump subunit AcrB; AltName: Full=AcrAB-TolC multidrug efflux pump subunit AcrB; AltName: Full=Acridine resistance protein B	367	C→G	Silent		■			■	
Cont52	pyoverdine sidechain peptide synthetase, partial	87	C→G	Silent		■				
Cont108	mammalian cell entry protein	19,461	C→A	Asp→Tyr				■		
Cont112	Carbamoyltransferase in large core OS assembly cluster	84,231	T→G	Asp→Ala			■			■
Cont43	TetR family transcriptional regulator	217,693	G→A	Gln→Stop	■			■		

Cont85	chloramphenicol acetyltransferase	23,702	A→C	Thr→Pro		▨			▨	
Cont75	MULTISPECIES: dehydrogenase [Pseudomonas],hypothetical protein PA0943 [Pseudomonas aeruginosa PAO1]	28,392	G→A	Trp→Stop		▨			▨	
Cont92	Full=UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase; AltName: Full=Protein EnvA; AltName: Full=UDP-3-O-acyl-GlcNAc deacetylase	94,358	A→G	Ile→Thr		▨			▨	
Cont27	outer membrane protein assembly complex, YaeT protein	75,983	T→C	Gln→Arg			▨			▨
Cont27	phosphoesterase	481,483	A→C	Thr→Pro			▨			▨
Cont42	RecName: Full=Protease HtpX; AltName: Full=Heat shock protein HtpX	290,119	A→G	Ser→Pro			▨			▨

Table S4C. Details of DIP (deletion, insertion and other polymorphisms) mutations in *P. aeruginosa* DP evolved populations. Mutations observed in control populations were removed to consider long-term exposure of LB media effect. Gray shading indicates populations with a mutation.

Contig	**Annotation**	**Gene position (Contig)**	**Mutation in contig**	**BAC(+)_1**	**BAC(+)_2**	**BAC(+)_3**	**BAC(-)_1**	**BAC(-)_2**	**BAC(-)_3**
Cont130	tolQ-type transporter	10,940	+TGGCGG	■			■		
Cont174	RecName: Full=Uroporphyrinogen-III C-methyltransferase; Short=Urogen III methylase; AltName: Full=SUMT; AltName: Full=Uroporphyrinogen III methylase; Short=UROM	25,207	Δ36 bp				■		
Cont125	peptidase S41	1,654	G→.	■			■		
Cont125	peptidase S41	1,537	+A		■			■	
Cont181	enoyl-CoA hydratase	1,397	Δ40 bp (large del)	■			■		
Cont38	TetR family transcriptional regulator	18,939	191 bp x 2	■					
Cont16	sugar-binding protein	19,119	A→.		■			■	
Cont162	hydrogenase expression protein HypA, multidrug efflux protein, RND efflux system, inner membrane transporter CmeB	21,318	Δ387 bp		■			■	
Cont162	hemolysin D, periplasmic multidrug efflux lipoprotein, RND multidrug efflux membrane fusion protein	19,001	C→.			■			■

	precursor, RND efflux system, membrane fusion protein CmeA					■			■
Cont184	phosphatidylglycerophosphatase A	767	Δ6 bp		■			■	
Cont19	acyl-CoA dehydrogenase	4,225	G→.		■			■	
Cont19	acyl-CoA dehydrogenase	4,226	C→.		■			■	
Cont67	PhoP/Q and low Mg2+ inducible outer membrane protein H1	857	Δ8 bp		■			■	
Cont201	lipopolysaccharide B-band O-antigen biosynthesis protein	29,039	G→.			■			■
Cont58	RecName: Full=Transcriptional activator protein LasR [Pseudomonas aeruginosa PAO1]	29,332	+15 bp				■		
Cont42	RecName: Full=DNA mismatch repair protein MutS	46,203	+9 bp						■
Cont42	spermidine dehydrogenase, SpdH	148,787	+C						■

Table S4D. Details of DIP (deletion, insertion and other polymorphisms) mutations in *P. aeruginosa* DPB evolved populations. Mutations observed in control populations were removed to consider long-term exposure of LB media effect. Black shading indicates populations with a mutation.

Contig	**Annotation**	**Gene position (Contig)**	**Mutation in contig**	**BAC(+)_1**	**BAC(+)_2**	**BAC(+)_3**	**BAC(-)_1**	**BAC(-)_2**	**BAC(-)_3**
Cont110	PmrA: two-component regulator system response regulator PmrA	94,728	+20 bp				■		
Cont98	Full=TPR repeat-containing protein PA4667	6,631	Δ4 bp		■			■	
Cont48	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	164,191	+12bp	■			■		
Cont112	histidine kinase	27,735	Δ9 bp	■			■		
Cont112	histidine kinase	27,867	Δ9 bp	■			■		
Cont115	peptidase S41	44,082	G→.	■			■		
Cont43	TetR family transcriptional regulator	217,721	+G		■			■	
Cont85	Full=Chloramphenicol acetyltransferase; AltName: Full=Xenobiotic acetyltransferase; Short=XAT	22,185	Δ1,817 bp	■			■		
Cont118	O-antigen acetylase	68,790	+G		■			■	
Cont118	O-antigen acetylase	70,620	C→.			■			■
Cont92	alpha-galactosidase	14,273	+C		■			■	
Cont92	RecName: Full=60 kDa chaperonin; AltName: Full=GroEL protein;	71,372	Δ36 bp			■			■

	AltName: Full=Protein Cpn60					■			■
Cont96	RecName: Full=Cytochrome c551 peroxidase; Short=CCP; Short=Cytochrome c peroxidase; Flags: Precursor	50,169	Δ32 bp		■			■	
Cont72	two-component sensor PhoQ	226,316	Δ92 bp			■			■
Cont123	nonCDS	804	219 bp x 2					■	
Cont55	TonB-denpendent receptor	7,536	+GCGCTGG					■	

Table S5. Subpopulational mutations in *pmrB* and *pmrA* in *P. aeruginosa* DP BAC(-) populations (Breseq polymorphism mode).

Comparison	**Contig**	**Annotation**	**Gene position (Contig)**	**Mutation in contig**	**Amino acid change**	**frequency**
BAC(-)_2_reads_vs_ancestor_contig	Cont53	*pmrA*	10,036	G→C	L(ancestor and BAC(+)_2) 59 V(BAC(-)_2)	26.80%
		pmrB	8,872	Δ7 bp		7.10%
		pmrB	8,249	C→T	G(ancestor and BAC(+)_2) 421 S(BAC(-)_2)	55.80%
BAC(-)_3_reads_vs_ancestor_contig		*pmrB*	9,449	A→G	F(ancestor and BAC(+)_3) 21 V(BAC(-)_3)	55.20%

Table S6A. Mean transcript abundance and log2 fold change of *arnBCADTEF* under BAC free condition (only LB) in *P. aeruginosa* DPB populations. AL: *P. aeruginosa* DPB ancestor in LB growth media; C2L: *P. aeruginosa* DPB_Control_2 in LB; BAC(+)_1L: *P. aeruginosa* DPB_BAC(+)_1 in LB; BAC(+)_2L: *P. aeruginosa* DPB BAC(+)_2 in LB; BAC(+)_3L: *P. aeruginosa* DPB BAC(+)_3 in LB.

AL_vs_C2L				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	-0.19	0.465671379	2881.36	0.95
arnC	-0.39	0.47302977	1451.45	0.86
arnA	-0.42	0.500156202	1981.62	0.85
arnD	-0.13	0.501352141	46.85	0.97
arnT (partial)	-0.13	0.442507489	41.99	0.97
arnT (partial)	-0.33	0.49133792	7.30	0.91
arnE	-0.52	0.404176235	0.62	NA
arnF	-0.95	0.492629556	0.91	NA
AL_vs_BAC(+)_1L				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	-0.73	0.431743475	2405.79	0.53
arnC	-1.13	0.472772597	1175.81	0.18
arnA	-0.59	0.504809163	1886.49	0.78
arnD	0.10	0.554740919	52.12	0.99
arnT (partial)	1.01	0.519615554	75.69	0.39
arnT (partial)	1.12	0.524817195	15.96	0.29
arnE	0.30	0.618855137	1.57	NA
arnF	1.04	0.612313641	3.52	0.53
AL_vs_BAC(+)_2L				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	0.32	0.533642396	3428.44	0.91
arnC	-0.58	0.569188667	1415.15	0.81
arnA	-0.55	0.570356139	1988.36	0.82
arnD	0.18	0.594907641	52.14	0.97
arnT (partial)	0.58	0.540764554	53.92	0.78
arnT (partial)	0.82	0.584488374	11.75	0.63
arnE	0.01	0.693819434	1.24	1.00
arnF	-1.07	0.71890835	1.07	0.60
AL_vs_BAC(+)_3L				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	0.46	0.452365733	3609.00	0.60
arnC	0.27	0.482877747	1820.99	0.81
arnA	0.73	0.506073653	3138.40	0.41
arnD	1.91	0.517494933	128.36	0.00
arnT (partial)	2.24	0.432234197	134.41	0.00

arnT (partial)	2.70	0.456141305	36.24	0.00
arnE	1.20	0.809027602	2.77	0.39
arnF	1.38	0.728107695	3.82	0.23

Table S6B. Mean transcript abundance and log2 fold change of *arnBCADTEF* under BAC exposure (LB+BAC) in *P. aeruginosa* DPB populations. AB: *P. aeruginosa* DPB ancestor in LB+BAC media; C2B: *P. aeruginosa* DPB Control_2 in LB+BAC; BAC(+)_1B: *P. aeruginosa* DPB BAC(+)_1 in LB+BAC; BAC(+)_2B: *P. aeruginosa* DPB BAC(+)_2 in LB+BAC; BAC(+)_3B: *P. aeruginosa* DPB BAC(+)_3 in LB+BAC.

AB_vs_C2B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	0.09	0.215104554	801.92	1.00
arnC	0.12	0.23495053	550.45	1.00
arnA	0.01	0.207432747	1599.01	1.00
arnD	-0.20	0.327154778	43.52	1.00
arnT (partial)	-0.01	0.302074495	67.17	1.00
arnT (partial)	-0.59	0.415935123	12.70	0.84
arnE	0.31	0.404163246	0.97	NA
arnF	0.12	0.453587471	1.20	NA
AB_vs_BAC(+)_1B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	1.28	0.23754236	1160.45	0.00
arnC	1.36	0.269060725	824.93	0.00
arnA	1.45	0.224670717	2606.73	0.00
arnD	1.14	0.31840902	65.64	0.00
arnT (partial)	1.79	0.304537468	134.99	0.00
arnT (partial)	0.79	0.435402146	19.35	0.24
arnE	0.49	0.730632955	0.87	NA
arnF	1.48	0.727945525	2.56	NA
AB_vs_BAC(+)_2B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	1.00	0.294281292	1088.14	0.01
arnC	1.24	0.236915578	825.38	0.00
arnA	1.14	0.297513844	2395.02	0.00
arnD	1.07	0.395549988	69.30	0.05
arnT (partial)	1.61	0.315054698	130.25	0.00
arnT (partial)	0.56	0.396600845	18.63	0.45
arnE	-0.13	0.586931586	0.37	NA
arnF	0.82	0.73034847	1.74	0.59
AB_vs_BAC(+)_3B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**

arnB	2.90	0.21317264	2516.05	0.00
arnC	3.15	0.195491069	1989.37	0.00
arnA	2.83	0.216985234	4967.34	0.00
arnD	2.72	0.250158093	136.69	0.00
arnT (partial)	2.88	0.241083376	218.91	0.00
arnT (partial)	1.94	0.368403034	30.47	0.00
arnE	1.84	0.828393669	1.90	NA
arnF	2.22	0.767476884	3.86	0.02

Table S6C. Mean transcript abundance and log2 fold change of *arnBCADTEF* under BAC exposure (LB+BAC) in *P. aeruginosa* DPB BAC(-)_1 and BAC(-)_2 compared with their ancestor. AB: *P. aeruginosa* DPB ancestor in LB+BAC media; BAC(-)_1B: *P. aeruginosa* DPB BAC(-)_1 in LB+BAC; BAC(-)_2B: *P. aeruginosa* DPB BAC(-)_2 in LB+BAC.

AB_vs_BAC(-)_1B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	0.09	0.219384811	782.96	0.87
arnC	0.44	0.222862261	607.57	0.20
arnA	0.12	0.2186852	1623.17	0.83
arnD	0.03	0.279480605	46.49	0.97
arnT (partial)	0.12	0.267812697	69.08	0.87
arnT (partial)	-0.09	0.411952553	15.07	0.93
arnE	-0.12	0.396253441	0.37	NA
arnF	0.14	0.564593045	1.13	NA
AB_vs_BAC(-)_2B				
	log2FoldChange	**lfcSE**	**baseMean**	**padj**
arnB	1.17	0.206620442	1439.22	0.00
arnC	1.72	0.205238777	1295.33	0.00
arnA	1.35	0.203125098	3225.66	0.00
arnD	1.19	0.265657064	88.47	0.00
arnT (partial)	1.52	0.240119011	151.20	0.00
arnT (partial)	0.80	0.359723774	25.34	0.09
arnE	0.48	0.794701467	0.88	NA
arnF	1.91	0.705407602	4.46	0.03

Table S7. Statistics of RNA-seq libraries. BAC(+)_2L_3 library was excluded for the analysis

	Total trimmed reads	**Minimum trimmed read length**	**Maximum trimmed read length**	**Mean trimmed read length**	**rRNA ratio (%)**	**non rRNA ratio (%)**
AB_1	4356082	50	251	149	1.44	98.56
AB_2	8141140	50	151	129	2.14	97.86
AB_3	7539488	50	151	129	0.79	99.21
AL_1	4011862	50	251	150	0.7	99.3
AL_2	6164494	50	151	114	0.83	99.17
AL_3	7641790	50	151	129	0.86	99.14
Control_2B_1	8957926	50	151	129	1.36	98.64
Control_2B_2	7750548	50	151	129	1.11	98.89
Control_2B_3	7685440	50	151	129	2.16	97.84
Control_2L_1	2741210	50	251	167	0.66	99.34
Control_2L_2	9891682	50	151	128	1.04	98.96
Control_2L_3	7469324	50	151	127	43.18	56.82
BAC(-)_1B_1	8025500	50	151	127	1.44	98.56
BAC(-)_1B_2	10073768	50	151	132	1.31	98.69
BAC(-)_1B_3	6256866	50	151	128	11.67	88.33
BAC(-)_2B_1	8949960	50	151	131	0.64	99.36
BAC(-)_2B_2	10280386	50	151	132	2.45	97.55
BAC(-)_2B_3	7658090	50	151	131	1.6	98.4
BAC(+)_1B_1	2461204	50	251	130	1.4	98.6
BAC(+)_1B_2	10681356	50	151	130	0.78	99.22
BAC(+)_1B_3	7222502	50	151	127	1.98	98.02
BAC(+)_1L_1	3194068	50	251	141	1.16	98.84
BAC(+)_1L_2	6712958	50	151	129	1.09	98.91
BAC(+)_1L_3	6435280	50	151	117	7.64	92.36
BAC(+)_2B_1	3284808	50	251	138	1.03	98.97
BAC(+)_2B_2	7898444	50	151	129	3.04	96.96
BAC(+)_2B_3	8581008	50	151	131	0.81	99.19
BAC(+)_2L_1	4010854	50	251	146	1.31	98.69
BAC(+)_2L_2	6788752	50	151	124	4.3	95.7
BAC(+)_2L_3	7386410	50	151	127	90.58	9.42
BAC(+)_3B_1	3123668	50	251	136	3.53	96.47
BAC(+)_3B_2	6892426	50	151	129	0.79	99.21
BAC(+)_3B_3	7881554	50	151	130	0.46	99.54
BAC(+)_3L_1	4514160	50	251	144	0.73	99.27
BAC(+)_3L_2	9863122	50	151	125	1	99
BAC(+)_3L_3	5876808	50	151	126	0.68	99.32