**Supplemental information:**

**Bacterial Strains and Media:**

*E. coli* DH5al*pir* was used for most cloning experiments due to the ability of blue/white screening, but plasmids were also constructed in Transformax EC100D *pir*+ (Lucigen; also enables blue/white screening but is StrR) and OneShot Pir1 (ThermoFisher; no blue/white screening). DH5al*pir* and Transformax EC100D *pir*+ were transformed with pTA-Mob [1] for some conjugation experiments, otherwise plasmids were transformed into SM10l*pir* for conjugation into target bacteria. *V. cholerae* strain O395 (StrR) [2] and *S. typhimurium* strain KK105 (TetR) [3] were used as recipients in conjugation. Concentrations of antibiotics used were: Strep 100 mg/ml, Tet 10 mg/ml, Amp 100 mg/ml, Kan 50 mg/ml, Cm 20 mg/ml (*E. coli*) or 2 mg/ml (*V. cholerae*), Erm 150 mg/ml. *E. coli, V. cholerae*, and *S. typhimurium* were grown in Luria Broth (LB) media (supplemented with 2 mM glutamine for *S. typhimurium*).

**Plasmid Construction:**

The allelic exchange plasmids were constructed in several steps. First, *Not*I and *Apa*I sites were added to the MCS of pUC118 [5] by site-directed mutagenesis using Quick Change (Stratagene), resulting in pKEK2094; the introduction of these sites was designed to maintain the coding frame of *lacZ*a. The MCS is shown in Fig. 1. Next, the *lacZ*a fragment from pKEK2094 was PCR amplified with primers lacZa F and lacZa R, and introduced by IVA cloning [7] into pDS132 [6] which was PCR amplified with primers pDS132R6K and pDS132 sacBp, resulting in pKEK2200. The KanR fragment from pKEK898 [8] was PCR amplified with primers Fnp F and pKEK898 KanR R, and introduced into pKEK2200 PCR amplified with primers pDS132Lac F and pDS132Lac R by IVA cloning, resulting in pKEK2201. The AmpR fragment from pUC118 was PCR amplified with primers AmpR F and AmpR R, digested with *Nde*I and *BamH*I, and ligated into pKEK2201 that had been digested with *Nde*I and *Bgl*II, to form pKEK2202.

The plasmid pKEK2297, described as an example used to delete the *flgH* gene from *V. cholerae*, was constructed by first PCR amplifying the two flanking fragments individually with flgHflank1 F + flgHflank1 R, and flgHflank2 F + flgHflank2 R, respectively. flgHflank1 F contains 16 bp sequence at the 5’ end complementary to universal priming site 1 (CGAGCTCGGTACCCGG), and flgHflank2 R contains 16 bp sequence at 5’ end complementary to universal priming site 2 (CTTGCATGCCTGCAGG). flgHflank1 R and flgHflank2 F contain 20 bp of complementary sequence that includes the *flgH* deletion. We typically use ThermoFisher Tm calculator website ([www.thermofisher.com](http://www.thermofisher.com)) and design primers with approximate Tm of 65°C. pKEK2200 was PCR amplified with primers universal 1 + universal 2, then digested for 1 h with *Dpn*I. We routinely achieve robust full-length vector (pKEK2200, pKEK2201, and pKEK2202) amplification with the following PCR conditions: KOD Hot Start Mastermix (Sigma Aldrich), 95°C 2 min, 19-30 cycles of 95°C 20 sec, 55°C 10 sec, 70°C 2 min. The three PCR fragments were mixed together (5 ml each) and transformed into DH5al*pir* carrying pTA-Mob that had been prepared for chemical transformation [9]. After 1 h incubation in LB at 37°C, cells were plated on LB Cm20 XGal40. Six plasmids from white colonies were screened, and all six had the correct digestion pattern.

The plasmid pKEK2287 used to inactivate *glnA* in *S. typhimurium* was constructed by PCR amplification of gDNA from KK27 [10] with primers STglnA F and STglnA R, which was then mixed with pKEK2200 that had been PCR amplified and DpnI digested as above, and then transformed into DH5al*pir* by chemical transformation.

**Strain construction:**

Plasmids were moved into target bacteria by conjugation, either from DH5al*pir*/pTA-Mob, Transformax EC100D *pir*+/pTA-Mob, or from SM10l*pir*. Mating was accomplished by cross-streaking and growth on LB agar, and transconjugants were then selected by growth on media containing the antibiotic resistance present on the mobilized plasmid and in the recipient bacteria (*V. cholerae* StrR CmR, *S. typhimurium* TetR CmR), indicating the plasmid had integrated into the chromosome by homologous recombination. Transconjugants were grown in LB for several cycles to allow the plasmid to excise from the chromosome by homologous recombination. In our hands it typically takes three cycles (i.e. inoculation at low cell density and outgrowth to stationary phase) of growth in LB to obtain sufficient colonies that have lost the plasmid, but this varies with different bacteria. The culture is then plated on LB agar (no NaCl) with 15% sucrose. Sucrose resistant colonies were screened for loss of antibiotic resistance associated with the plasmid; in our hands all sucrose resistant colonies are also CmS, indicating they have all lost the plasmid. In the examples in this report we analyzed SucR CmS colonies for the phenotypes associated with mutant genotypes (*flgH* = reduced motility; *glnA* = glutamine auxotrophy on minimal media). The chromosomal mutations were confirmed by PCR and sequencing.

**Table S1 – Oligonucleotides used in this study.**

|  |  |
| --- | --- |
| **Name** | **Sequence** |
| Universal 1 | **CCGGGTACCGAGCTCG**AATTC |
| Universal 2 | **CCTGCAGGCATGCAAG**CTT |
| pDS132 sacBp  | GATCCTTTTTAACCCATCACATATACCTGCCG |
| pDS132R6K  | CCATGTCAGCCGTTAAGTGTTCCTGTG |
| lacZa F  | TGGGTTAAAAAGGATCAGCGCCCAATACGCAAACC |
| lacZa R  | TTAACGGCTGACATGGTTAATGCGCCGCTACAGGG |
| pDS132Lac R  | CCGCTTACAGACAAGCTGTGACCG  |
| pDS132Lac F  | AGATCTGCTCCTGCCCTATGGGATTCACC |
| Fnp F | GCTTGTCTGTAAGCGGTTTGGGTTGTCACTCATCGTATTTGG |
| pKEK898 KanR R  | GGGCAGGAGCAGATCTTTAGAAAAACTCATCGAGCATCAAATGAAAC  |
| AmpR F | GCGCCATATGAGTATTCAACATTTCCGTGTCGCCC |
| AmpR R  | GCGCGGATCCTTACCAATGCTTAATCAGTGAGGCACCT |
| flgHflank1 F  | **CGAGCTCGGTACCCGG**ATTGCGAACGCCAATACGTTTG |
| flgHflank1 R  | TCGAGTTACGCCATGGCTTAGTTAGCAATTACAGTTGTTGGTTAAC |
| flgHflank2 F  | TAAGCCATGGCGTAACTCGAGCCGCTGGGCTAGTTTATTG |
| flgHflank2 R  | **CTTGCATGCCTGCAGG**TTCACCATGTTTTCTTTGCGTGACCG |
| flgH screen F | GGTTTAGAGCCGGTTGGACAAA |
| flgH screen R | TAAAAGGTGTCGACTCGCCAG  |
| STglnA F | **CGAGCTCGGTACCCGG**agatctgcatctgcaacggac |
| STglnA R | **CTTGCATGCCTGCAGG**cagtacttgctcaatttgttccgg |
| glnA screen F | GACCAATCCGGGAGAGTACAAG |
| glnA screen R | CGATTAAACGCTGTAGTACAGCTC |

**Table S2 – Strains and plasmids used in this study.**

|  |  |  |
| --- | --- | --- |
| **Strain** | **Description** | **Reference** |
| DH5α λ *pir* | *E. coli* |  |
| SM10 λ *pir* | *E. coli* | [12] |
| EC100D *pir*+ | *E. coli* | Lucigen |
| OneShot pir-1 | *E. coli*  | ThermoFisher |
| O395 | *V. cholerae* wildtype | [2] |
| KKV3177 | D*flgH V. cholerae* | This study |
| ATCC14028 | *S. typhimurium* wildtype | ATCC |
| KK802 | D*glnA S. typhimurium* | This study |
| **Plasmid** | **Description** | **Reference** |
| pDS132 | Sucrose-counterselectable suicide vector; CmR | [6] |
| pUC118 | High copy blue/white cloning vector; AmpR | [5] |
| pKEK2094 | pUC118 with *Not*I, *Apa*I sites added to MCS  | This study |
| pKEK2200 | Sucrose-counterselectable blue/white suicide vector; CmR | This study |
| pKEK2202 | Sucrose-counterselectable blue/white suicide vector; KanR | This study |
| pKEK2203 | Sucrose-counterselectable blue/white suicide vector; AmpR | This study |
| pKEK2297 | D*flgH* in pKEK2200 | This study |
| pKEK2287 | D*glnA* in pKEK2200 | This study |



**Fig S1:** PCR confirmation of *V. cholerae* and *S. typhimurium* mutants constructed with pKEK2200. The length of select DNA size markers (in kbp) is on left. gDNA from O395 (WT) and KKV3177 (D*flgH*) were PCR amplified with flgHscreen F & flgHscreen R, KKV3177 shows the expected 786 bp deletion. gDNA from ATCC14028 (WT) and KK802 (D*glnA*) were PCR amplified with glnAscreen F & glnAscreen R, KK802 shows the expected 267 bp deletion.

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