Single-Stranded DNA-Binding Protein and Exogenous RecBCD Inhibitors Enhance Phage-Derived Homologous Recombination in *Pseudomonas*

**HIGHLIGHTS**

- The BAS operon is a generic recombineering system for *Pseudomonas* species.
- Single-stranded DNA-binding proteins (SSBs) can stimulate homologous recombination.
- The heterologous gam genes can inhibit RecBCD function in *Pseudomonas*.

---

Jia Yin, Wentao Zheng, Yunsheng Gao, ..., A. Francis Stewart, Youming Zhang, Jun Fu

francis.stewart@tu-dresden.de (A.F.S.)
zhangyouming@sdu.edu.cn (Y.Z.)
fujun@sdu.edu.cn (J.F.)

Yin et al., iScience 14, 1–14
April 26, 2019 © 2019 The Author(s).
https://doi.org/10.1016/j.isci.2019.03.007
Single-Stranded DNA-Binding Protein and Exogenous RecBCD Inhibitors Enhance Phage-Derived Homologous Recombination in Pseudomonas

Jia Yin,1,2,5 Wentao Zheng,1,5 Yunsheng Gao,1 Chanjuan Jiang,1 Hongbo Shi,1 Xiaotong Diao,1 Shanshan Li,1 Hanna Chen,2 Hailong Wang,1 Ruijuan Li,1 Aijing Li,1 Liqiu Xia,2 Yulong Yin,2,3 A. Francis Stewart,4,* Youming Zhang,1,* and Jun Fu1,4,*

SUMMARY

The limited efficiency of the available tools for genetic manipulation of Pseudomonas limits fundamental research and utilization of this genus. We explored the properties of a lambda Red-like operon (BAS) from Pseudomonas aeruginosa phage Ab31 and a Rac bacteriophage RecET-like operon (RecTEPsy) from Pseudomonas syringae pv. syringae B728a. Compared with RecTEPsy, the BAS operon was functional at a higher temperature indicating potential to be a generic system for Pseudomonas. Owing to the lack of RecBCD inhibitor in the BAS operon, we added Redγ or Pluγ and found increased recombineering efficiencies in P. aeruginosa and Pseudomonas fluorescens but not in Pseudomonas putida and P. syringae. Overexpression of single-stranded DNA-binding protein enhanced recombineering in several contexts including RecET recombination in E. coli. The utility of these systems was demonstrated by engineering P. aeruginosa genomes to create an attenuated rhamnolipid producer. Our work enhances the potential for functional genomics in Pseudomonas.

INTRODUCTION

Recombinant DNA technology in Escherichia coli (E. coli) has been greatly boosted by bacteriophage-encoded recombination systems (Muyrers et al., 1999; Zhang et al., 1998, 2000). A decisive advantage of this technology, termed recombinogenic engineering or recombineering, is the easy incorporation of the flanking short homology sequences required for homologous recombination into synthetic oligonucleotides. Oligonucleotides and linear dsDNAs with homology arms as short as 35 nucleotides have been exploited in many ways for point mutations, deletions, insertions, and subcloning with base pair precision regardless of the size of the target DNA (Murphy, 1998; Muyrers et al., 1999; Zhang et al., 1998, 2000).

In E. coli, recombineering utilizes the Redα, Redβ, and Redγ proteins from the lambda phage Red operon or RecE and RecT from the Rac prophage (Fu et al., 2012; Zhang et al., 1998). Redα and RecE are 5’-3’ exo- nucleases that generate single-stranded DNA (ssDNA) intermediates (Maresca et al., 2010). Redβ and RecT are single-stranded DNA-annealing proteins (SSAPs) that bind to ssDNA to promote the search for the complementary sequence (Erler et al., 2009). The Redα/Redβ and RecE/RecT phage pairs each include a specific protein-protein interaction, which is required for double-stranded DNA (dsDNA) homologous recombination (Muyrers et al., 2000).

Redy forms a dimer to mimic DNA that binds to and inhibits the exonuclease and helicase activities of the RecBCD complex (Court et al., 2007; Murphy, 1991), which aggressively degrades linear dsDNA (Court et al., 2007; Wang et al., 2006). The RecET operon does not appear to have a Redy equivalent. However, the inclusion of Redy with RecE/RecT increases homologous recombination efficiency via increased persistence of the linear dsDNA substrates (Fu et al., 2012; Zhang et al., 1998). The significance of a RecBCD inhibitor for homologous recombination efficiency was also demonstrated in our study describing Photorhabdus luminescens Pluy for the development of a recombineering system for Photorhabdus and Xenorhabdus. We found that Pluy could inhibit the RecBCD complex in both Photorhabdus and E. coli (Yin et al., 2015).

The Red system has been adapted to precisely and fluently edit the genome of not only E. coli but also closely related bacteria Salmonella enterica (Bunny et al., 2002), Yersinia pseudotuberculosis (Derbise et al., 2005), and Yersinia pestis (Leplae et al., 2008). The RecET system has been applied to precisely and fluently edit the genome of not only E. coli but also closely related bacteria Salmonella enterica (Bunny et al., 2002), Yersinia pseudotuberculosis (Derbise et al., 2005), and Yersinia pestis (Leplae et al., 2008).
et al., 2003), *Shigella* (Beloin et al., 2003), *Serratia* (Rossi et al., 2003), and *Escherichia albertii* (Egan et al., 2016). However, its wider application has been limited by apparent host specificities (Yin et al., 2015).

Consequently endogenous SSAPs alone or together with partner exonucleases have been used for oligo repair or cassette insertion in *Mycobacterium tuberculosis* (Van Kessel and Hatfull, 2006), *Lactococcus lactis* (Pijkeren and Britton, 2012), *Lactococcus reuteri* (Pijkeren et al., 2012), *Clostridium acetobutylicum* (Dong et al., 2013), *Lactobacillus plantarum* (Yang et al., 2015), *Bacillus subtilis* (Sun et al., 2015), *P. luminescens*, and *Xenorhabdus stockiae* (Yin et al., 2015).

*Pseudomonas* is a gram-negative, aerobic rod that belongs to the bacterial family Pseudomonadaceae (EUZeBY, 1997). The best characterized species include the opportunistic human pathogen *Pseudomonas aeruginosa* (Stover et al., 2000), the plant pathogen *Pseudomonas syringae* (Xin and He, 2013), the plant growth-promoting *Pseudomonas fluorescens* (Paulsen et al., 2005), and the soil bacterium *Pseudomonas putida*, which is used in bioremediation (Gomes et al., 2005) and biocontrol (Validov et al., 2007). The members of this genus demonstrate a great deal of metabolic diversity (Aditi et al., 2017).

Here, we report the development of recombineering systems for *Pseudomonas* based on two host-specific phage protein-encoding operons from *P. aeruginosa* phage Ab31 and *P. syringae*. BLAST analysis suggested that Orf38, Orf37, and Orf36 are analogs of Redβ, Redα, and single-stranded DNA-binding protein (SSB), respectively. Recombineering experiments indicated that Redy or Pluβ worked in some *Pseudomonas* species and SSB significantly increased efficiency. SSBs are often found in recombinase-encoding operons from various phages (Szczepańska, 2009). Because proteins in the same operon are usually functionally associated, we were particularly interested in evaluating the contribution of orf36/S to phage recombinase-mediated homologous recombination. Using these systems, we efficiently modified *Pseudomonas* genomes including gene deletions and insertions. In particular, a highly attenuated rhamnolipid producer was obtained after deleting pathogenic factors and overexpressing rhlAB and estA in *P. aeruginosa*.

**RESULTS**

**Endogenous Phage exo/SSAP Protein Pairs in Pseudomonas**

We looked for candidate DNA recombination proteins in *Pseudomonas* and *Pseudomonas* phage genomes using BLAST with the coding sequences of λ Redβ, rac RecT, or Pluβ as queries in a non-redundant protein sequence database. Two operons including an exonuclease (exo) and SSAP were identified. One was RecTEPsy from *P. syringae*, which was previously developed as a recombineering system for use in *P. syringae* (SwINGLE et al., 2010). The second operon, from *P. aeruginosa* phage vB_PaeP_Tr60_Ab31, encoded three proteins including a candidate SSAP (orf38, here named B) that was 62% identical to Redβ over its 177-amino acid sequence (Figure 1) and was adjacent to a candidate exonuclease (orf37, here named A; Figure 1), which showed significant similarity to Redα (sequence identity of 32% in a 195-amino acid region). The next coding region, orf36 (here named S), showed significant similarity to SSBs (sequence identity of 54% across 148 amino acids of the *E. coli* SSB; Figure 1). SSBs are often found...
in recombinase-encoding operons from various phages (Szczepańska, 2009). Because proteins in the same operon are usually functionally associated, we evaluated the contribution of orf36/S to phage recombinase-mediated homologous recombination and we named this candidate recombineering operon, BAS.

In gram-negative bacteria, RecBCD, which is the major exonuclease in E. coli, is highly conserved (Table S5). Notably neither RecTEPsy nor BAS operons contained a homolog of Redg or Plug, which are inhibitors of RecBCD exonuclease activity. Because co-expression of Redy with RedPy or RecET significantly increases recombineering efficiency (Fu et al., 2012), the addition of Redy or Plu to the RecTEPsy and BAS operons were tested for their abilities to increase recombineering efficiency.

Critical Time Points for Electroporation of Pseudomonas

In E. coli, transformation efficiency determines optimal recombineering (Sharan et al., 2009). When E. coli DH10B-derived cells are used, the recombinant proteins are usually induced when the cells enter log phase growth (OD_{600} = 0.30–0.35). After two cell divisions, at OD_{600} = 0.70–0.80, the electrocompetent cells are prepared (Fu et al., 2010). This principle was also verified by recombineering in Photorhabdus (Yin et al., 2015). To optimize protocols for Pseudomonas, we first plotted the growth of the four Pseudomonas strains at 30°C. Overnight cultures were diluted to OD_{600} = 0.085 to start the growth-monitoring cultures. After approximately 2 h, P. syringae and P. aeruginosa cultures entered the log phase and the plasmid pBBR1-Rha-GFP-kan was transformed into electrocompetent cells prepared at different time points (Figure 2). We found that cells prepared at 4 and 2.5 h, respectively, yielded the most transformants (Figures 2B and 2D). The same test was performed for P. fluorescens (Figures S2A and S2B) and P. putida (Figures S2C and S2D). Thereby we established the time points for induction and electrocompetent preparation for each strain (Table S6).

Efficiency of Recombineering Systems in Pseudomonas

We compared the RecTEPsy, BAS, Redyβx, and Pluyβx systems in four Pseudomonas strains. Pluy or Redy was added to the RecTEPsy and BAS operons to generate four more candidate systems: PluyTePsy, RedyTePsy, PluyBAS, and RedyBAS. The eight expression plasmids were transformed into the four Pseudomonas strains, followed by plasmid DNA extraction and restriction analysis for confirmation. The expression plasmids were based on a broad host range origin (pBBR1) (Antoine and Locht, 1992), and
Figure 3. Recombineering with Different Protein Combinations in Different Pseudomonas Species

(A) Diagram of the recombineering assay. A PCR product carrying a gentamycin resistance gene (Genta) flanked by 100-bp homology arms (thick lines) was integrated into the expression plasmid in place of the artificial operon of the recombination system.

(B) Results from the recombineering assay in P. fluorescens upon the expression of Plu\(\gamma\)β\(\gamma\), Red\(\gamma\)β\(\gamma\), TE\(\gamma\), Plu\(\gamma\)TE\(\gamma\), Red\(\gamma\)TE\(\gamma\), BAS, Plu\(\gamma\)BAS, and Red\(\gamma\)BAS at 30°C.

(C) Results from P. syringae, which was set up as described in (B).

(D) Results from P. putida, which was set up as described in (B), with the exception that Red\(\gamma\)TE\(\gamma\) was missing owing to the failure of transformation.

(E) Results from P. aeruginosa at 30°C, which was set up as described in (B), and the correct rates are indicated at the top of each column.

(F) Results from P. aeruginosa at 30°C, which was calculated from the correct rates of (E), and the y axis represents the number of correct colony.

(G) Results from P. aeruginosa at 37°C, which was set up as described in (B), and the correct rates are indicated at the top of each column.

(H) Results from P. aeruginosa at 37°C, which was calculated from the correct rates of (E), and the y axis represents the number of correct colony.
the rhaR-rhaS P$_{rha}$ promoter (Egan and Schleif, 1993, 1994) was used for inducible expression of the recombination operons.

To compare the recombineering efficiency of different systems in one species, all the electroporation was carried out using competent cells prepared from a 1.3 mL fresh culture that are adjusted to the same cell density. After electroporation, 1 mL Luria-Bertani (LB) medium was added for recovery. The data presented are exactly the result from one electroporation, and this methodology was utilized in our previous study (Fu et al., 2012; Yin et al., 2015).

We used an assay based on the integration of a PCR product containing gentamycin resistance gene flanked by 100 bp homology arms into the pBBR1 expression plasmid to obtain the plasmid product, pBBR1-Kan-Genta (Figure 3A). Results are based on counting gentamycin-resistant colonies followed by verification of pBBR1-Kan-Genta by restriction analysis.

P. fluorescens, P. syringae, and P. putida were grown and tested at 30°C (Figures 3B–3D). Eight recombinants of pBBR1-Kan-Genta were verified by restriction analysis and found to be 100% in all three strains. Expression of PluG and RedY enhanced recombination in P. fluorescens but appeared to impair it in P. putida possibly because the expression of PluG or RedY had a toxic effect. Indeed, we could not cultivate P. putida carrying the RedYTEP$_{sy}$ expression plasmid. In all three strains, both TEP$_{sy}$ and BAS systems worked better than RedYβx and PluGβx.

A different outcome was observed in P. aeruginosa where the acquisition of gentamycin resistance, possibly due to intrinsic multidrug resistance mechanisms (Savoia, 2014), did not faithfully reflect recombination into pBBR1. Consequently, both gentamycin-resistant colonies and the correct recombination rates are presented in Figures 3E–3H. Because P. aeruginosa normally grows at 37°C, we tested recombination at both 30°C and 37°C. At 30°C, PluG significantly increased the efficiency of both the TEP$_{sy}$ and BAS systems, but it failed to have an effect at 37°C indicating thermolability. In contrast, RedY increased the efficiency of BAS at both 30°C and 37°C. As with the other three strains, the PluGTEP$_{sy}$, PluGβx, and RedYβx systems were superior to RedYβx and PluGβx. Notably, the correct ratio of PluGTEP$_{sy}$ at 30°C was 92%, which was the most efficient of all variations tested in P. aeruginosa. However, PluGTEP$_{sy}$ showed no activity at 37°C and RedYβx presented the best performance (Figures 3E–3H). We suggest that PluG and TEP$_{sy}$ are inactive above 30°C and RedY and BAS are active at 37°C. These temperature optima concord with the origins of these proteins.

**Functional Dissection of BAS**

Based on the above evaluation, the BAS system, with or without RedY, can be used for genome engineering in various Pseudomonas strains. Because the single-stranded DNA-binding protein S is the unusual aspect of this operon, we further investigated its contribution to recombineering first in E. coli where neither BA nor BAS functioned well without RedY. However, in the presence of RedY, S promoted recombineering efficiency about 4-fold (Figure 4A). A similar effect was found in P. aeruginosa (Figure 4B), whereas in P. syringae, RedY stimulated recombination in the absence of S but impaired it in its presence (Figure 4C).

To further investigate the effect of S, we evaluated its effect of Red- and RecET-mediated recombination, which differ mechanistically. RedYβx requires a replicating substrate and acts at the replication fork (Maresca et al., 2010), whereas full-length RecE/RecT recombinates two linear DNAs before replication (Fu et al., 2012). We term these two mechanisms linear plus circular homologous recombination (LCHR) and linear plus linear homologous recombination (LLHR). Addition of S to Red LCHR has a small positive effect, whereas it substantially enhances RecET LCHR (Figure 4D). Conversely, S does not convey LLHR capacity onto Red but substantially enhances RecET LLHR (Figure 4E). Consequently, we tried two more SSBs, which are from E. coli and F plasmid, and both also enhanced RecET LLHR (Figure 4F).
Optimization of Recombineering in *Pseudomonas*

After identifying the optimal recombineering configurations for each *Pseudomonas* strain, i.e., Plu-TEPsy for *P. aeruginosa* at 30°C, RedyBAS for *P. aeruginosa* at 37°C, BAS for *P. putida* and *P. syringae*, and RedyTEPsy or RedyBAS for *P. fluorescens*, we further optimized the protocols. Apart from electrocompetent cell preparation, the amount of DNA to be transformed and the length of its homology arm to the target play large roles in determining efficiency (Fu et al., 2012; Yin et al., 2015). We titrated the DNA amount from 100 ng to 2 μg. It was observed that 1 μg DNA was sufficient for *P. fluorescens*, *P. syringae*, and *P. putida* (Figures S3A, S3C, and S3E). However, *P. aeruginosa* required 1.5 μg of DNA to achieve a decent efficiency (Figure S3G). For all three strains, the saturation amount of DNA was 1.5 μg. The length of the homology arm was tested across a range from 30 to 150 bp. One hundred base pairs were sufficient for *P. fluorescens* (Figure S3B) and *P. putida* (Figure S3F). *P. syringae* (Figure S3D) and *P. aeruginosa*...
Recombineering Pseudomonas Genomes

To validate the methods, we designed several genome engineering exercises beginning with the construction of an attenuated *P. aeruginosa* PAO1 mutant. *P. aeruginosa* produces a number of virulence factors, including exotoxin A (McEwan et al., 2012), pyocyanin (Yang et al., 2016), elastase (McIver et al., 1991), and periplasmic glucans (Mahajan-Miklos et al., 1999), which prevent the safe use of this strain for the production of rhamnolipids. As diagrammed in Figure 5A, a number of gene knockouts were performed by electroporating a PCR product containing a gentamycin resistance gene flanked by lox71/lox66 sites and 75-bp homology arms. The *aroA* gene (1917 bp) encodes 3-phosphoshikimate 1-carboxyvinyltransferase, which is essential for the synthesis of aromatic amino acids. Deletion of *aroA* can completely prevent *P. aeruginosa* infection of mammalian cells (Priebe et al., 2002). The *phnAB* (2172bp) gene is involved in pyocyanin biosynthesis (Lau et al., 2004). The *lasB* gene (1497 bp) encodes elastase (McIver et al., 1991). The *mdoH* gene (2586 bp) is required for the biosynthesis of periplasmic glucans (Mahajan-Miklos et al., 1999). To knock out these genes, each electroporation yielded approximately 100 gentamycin-resistant colonies when Plu*TEPsy* was expressed, and all the clones were correct. We also used the RedyBAS system and noticed approximately 1000 small and 100 large gentamycin-resistant colonies, but only about half of
the large colonies were correct according to colony PCR (Figures S5B, 5D, 5F, and 5H). To generate a selectable marker-free P. aeruginosa PAO1 mutant, Cre catalyzed the excision of the lox-flanked DNA (Figures 5C, 5E, 5G, and 5I).

Pseudomonas exotoxin A is a single-chain toxin with three structural domains that inhibit protein synthesis in eukaryotic cells by catalyzing ADP ribosylation of elongation factor 2. The amino-terminal domain I is involved in eukaryotic cell recognition (Jinno et al., 1988), the central domain II may be involved in the translocation function of the protein (Siegal et al., 1989), and the carboxy-terminal domain III has a clef t that is proposed to be the enzyme active site (Carroll and Collier, 1987). Glu-578 in domain III has been identified as an active site residue for nicotinamide adenine dinucleotide binding (Carroll and Collier, 1987). To evaluate the ability of the recombineering system to achieve a seamless mutation, we aimed to mutate this residue. A single-nucleotide substitution of ToxA (GAG Glu-578 codon to GAC Asp) was achieved by two rounds of recombineering (Figure S4A). First, a PCR product with a gentamycin resistance and counter-selection (SacB) genes flanked by 75-bp homology arms was introduced into P. aeruginosa expressing PluvTEp aer. After the gentamycin-resistant clones were verified by colony PCR (Figure S4B), SacB function was tested (Figure S5). In the second round of recombineering, a point mutation (GAG Glu-578 codon to GAC Asp) was introduced using 121-bp oligonucleotide with 60-bp homology arms either side of the point mutation. The reaction was mediated by PluvTEp aer, and the mutant strain was screened on LB plates containing 10% sucrose. Successful site-directed mutagenesis was confirmed by colony PCR (Figure S4C) and sequencing (Figure S4D).

The BAS system was also successfully applied to genome engineering of P. syringae pv. tomato str. DC3000 by placing a promoter (Pgen 2) in front of a silent gene cluster for a secondary metabolite pathway. Insertion of the promoter was efficient. Each electroporation yielded about 120 gentamicin-resistant colonies. Three colonies were verified by colony PCR (Figure S6A), and all of them were correct. However, we could not detect any new compound produced by the engineered strain (data not shown). We also engineered the P. fluorescens genome with the Red从来 system. The retS gene of P. fluorescens was knocked out, and the mutant strain was confirmed by colony PCR (Figure S6B). Each electroporation yielded approximately 20 gentamicin-resistant colonies, and all of them were correct. The hybrid sensor kinase (RetS) negatively controls the production of antibiotics, including 2,4-diacyltrophloroglucinol, pyrolnitrin, and pyoluteorin, in P. fluorescens (Zhang et al., 2015). Genetic inactivation of retS results in enhanced biocontrol capacity of the strain (Zhang et al., 2015).

Using the Attenuated P. aeruginosa PAO1 Mutant for Rhamnolipid Production

Although much effort has been invested in heterologous expression of rhlAB, the pathogenic strain P. aeruginosa PAO1 remains the best producer of rhamnolipids (Zhao et al., 2015). To evaluate the safety of an attenuated P. aeruginosa PAO1 strain, we examined their virulence in mice and C. elegans models. The 50% lethal dose for wild-type PAO1 given by intraperitoneal (i.p.) injection was 4 × 10⁵ colony-forming unit (CFU). However, no toxic effects were observed with i.p. doses of the attenuated mutant PAO1aroA up to 1 × 10⁷ CFU (Figure 6A). In contrast, the PABMT mutant (phnAB-, lasB-, mdoH-, and toxA-) was still pathogenic to BALB/c mice (Table S7) and postponed the egg stage in C. elegans (Figure S7).

The PAO1aroA mutant is auxotrophic for aromatic amino acids and must be cultivated with aromatic amino acid supplement. Under these conditions, the wild-type PAO1 strain entered log phase in 2 h, but the PAO1aroA mutant strain grew more slowly, entering log phase in 5 h (Figure S8). Nevertheless, when we compared rhamnolipid production after 48 h of fermentation, there was no significant difference between the wild-type PAO1 and PAO1aroA mutant strains (Figure 6B).

To increase rhamnolipid production in the PAO1aroA mutant, we introduced a plasmid carrying rhlAB, which participates in the biosynthesis of this biosurfactant (Ochsner et al., 1994), and the estA gene, which encodes an autotransporter protein located in the outer membrane (Wilhelm et al., 2007) (Figure S9). Overexpression of rhlAB and estA in wild-type PAO1 and the PAO1aroA mutant increased the yield of rhamnolipids (Figure 6B). Using a toluene emulsion assay (Biggins et al., 2014), we tested the emulsive potential of the PAO1aroA mutant in the presence of rhlAB and estA. Visible emulsions formed in the supernatants of both wild-type PAO1 and the PAO1aroA mutant (Figure 6C). We also confirmed the stability of the expression plasmid pBBR1-estA-genta-rhlAB, which replicated in the PAO1aroA mutant without antibiotic selection (Figure 6D). Thus a safe rhamnolipid producer for industrial fermentation has been established.
Although the Red system has been applied to engineer the genome of *E. coli* and a number of genetically close species, apparent host-specific factors limit its wider application (Yin et al., 2015). Consequently, we and others have searched for other phage exonuclease/SSAP pairs associated with genetically distant bacterium. This includes the prophage RecETPsy system, which has been used in *P. syringae* so far (Swingle et al., 2010).

Several attempts of recombineering in *P. putida* have been recently reported. However, the enzymes are lambda Red (Chen et al., 2016; Cook et al., 2018; Luo et al., 2016) and rac RecET (Choi et al., 2018), or an SSAP without an accompanying exonuclease (Aparicio et al., 2016, 2018; Ricaurte et al., 2018). It is well known that the use of SSAPs alone is only effective with single-stranded oligonucleotides and will not mediate the dsDNA homologous recombination events.

**Figure 6. Overexpression of rhlAB and estA in the PAO1 Mutant for Rhamnolipid Production**

(A) The survival rate of BALB/c mice after intraperitoneal injection of wild-type PAO1 and the PAO1aroA mutant strain (1*10^8 CFU).

(B) Rhamnolipid production in wild-type PAO1 and the PAO1aroA mutant from a 2-day fermentation. The statistical analysis used is Student's t test. *p < 0.05, **p < 0.01.

(C) Emulsification activity of cell culture supernatants. Equal parts of toluene and supernatant were combined in a glass vial, vortexed vigorously, and rested for 2 h.

(D) Plasmid stability in the PAO1aroA mutant. The tested plasmid was pBBRI-estA-genta-rhlAB. This strain was cultured in Murashige and Skoog (MS) medium supplemented with aromatic amino acids without antibiotic, and the number of cells was counted on LB plates with and without gentamycin every day. The ratio of gentamycin-resistant clones to total cells was calculated. Data represent the mean ± SD from three independent experiments.

**DISCUSSION**

Although the Red system has been applied to engineer the genome of *E. coli* and a number of genetically close species, apparent host-specific factors limit its wider application (Yin et al., 2015). Consequently, we and others have searched for other phage exonuclease/SSAP pairs associated with genetically distant bacterium. This includes the prophage RecETPsy system, which has been used in *P. syringae* so far (Swingle et al., 2010).

*Pseudomonas* is a large genus including *P. aeruginosa, P. chlororaphis, P. fluorescens, P. putida, P. stutzeri, P. syringae*, and a large number of *Incertae sedis*. In a search for recombineering solutions to cover this broad range, we worked with representatives from four groups, namely, *P. aeruginosa, P. fluorescens, P. putida*, and *P. syringae*. In addition to RecETPsy, we identified another operon in the *Pseudomonas* phage vB_PaeP_Tr60_Ab31 (Latino et al., 2014) (Figure 1). These operons differ from the *E. coli* lambda Red operon (RedYβx) or our previously reported *P. luminescens* operon (Pluyβx) (Yin et al., 2015) because they do not appear to include a Redy/Pluy-type inhibitor of the major host exonuclease RecBCD, which protects linear dsDNA ends from degradation and thereby facilitates recombination. Therefore we added Pluy and Redy to the TEPsy and BAS systems to evaluate their impact...
on recombineering. Owing to apparent toxicity and temperature optima, we did not arrive at a single optimal configuration for all four Pseudomonas species. In P. aeruginosa PluyTE$_{py}$, delivered the best results at 30°C, whereas RedyBAS was the configuration for 37°C. Furthermore, Redy and Pluy increased recombineering efficiency in P. aeruginosa and P. fluorescens, but Redy was deleterious in P. putida and P. syringae. We suggest that Redy likely alters the function of the RecBCD complex in E. coli, P. aeruginosa, and P. fluorescens in the same manner by temporarily blocking its exonuclease activity. However, the interaction between Redy and RecBCD in P. syringae or P. putida might proceed differently, potentially to have an effect on RecBCD-mediated genome repair.

Consequently, after evaluating different configurations in four Pseudomonas species, we found that the BAS system was functional in all four tested Pseudomonas strains and so has the potential to be a generic system for Pseudomonas after testing for Pluy or Redy functionality.

We report the usefulness of an SSB in a recombineering system. The three-element BAS and four-element hybrid RedyBAS systems are useful genome engineering tools in certain Pseudomonas strains. Consequently, we were motivated to explore the utility of S, the phage Ab31 SSB, in both established recombineering paradigms, Red and RecET in LCHR and LLHR applications.

In E. coli, S clearly contributed to BA recombination. Addition of S had little impact on Red recombination. However, it substantially enhanced RecET in both LCHR and LLHR assays (Figures 4D and 4E).

SSBs play crucial roles in DNA replication, recombination, DNA damage signaling, and repair in all organisms (Yang et al., 2013). In addition, SSBs can protect ssDNA from further degradation after 5’-3’ exonuclease resection (Meyer and Laine, 1990). SSB also forms a protein interaction platform by recruiting DNA replication and recombination enzymes, such as exonuclease I (Lu et al., 2011) and RecJ (Han et al., 2006). Biochemical studies of different recombination systems have revealed that SSB binds to and removes secondary structures from ssDNA during the presynapsis stage (Kowalczykowski and Krupp, 1987), which facilitates homologous strand pairing. Therefore, an SSB could potentially contribute to recombineering in three ways, namely, protection of ssDNAs involved in recombination from degradation, interaction(s) with the recombination machinery, or removal of secondary structures in ssDNA intermediates. We designed an experiment to test this third possibility to determine if homologous recombination efficiency was affected by secondary structure of the homology arm and whether an SSB could ameliorate the impact. However, on comparison of a homology arm with notable secondary structure to an arm with moderate secondary structure, there was no bias with or without SSB overexpression in the recombineering host (Figures 7A–7C). Although further studies are necessary to elucidate the biological role of SSB in recombineering, we suggest that the variable contributions of a RecBCD inhibitor or an SSB in different contexts reflect different recombination susceptibilities to double- or single-stranded exonucleases.

Based on the RedyBAS and PluyTE$_{py}$ recombineering systems, we constructed two P. aeruginosa mutants, PABMT and PAO1aroA. PABMT could postpone the egg stage for C. elegans but was still pathogenic to BALB/c mice, suggesting the presence of additional pathogenic factors. PAO1aroA is a highly attenuated strain (Figure 6A) with potential for several applications, such as live vaccines (Priebe et al., 2002). In the present study, the highly attenuated mutant was used to produce rhamnolipid biosurfactants. Rhamnolipids are surface-active secondary metabolites produced by P. aeruginosa or related species in the stationary phase (Zhang et al., 2012). In the last decade, these molecules have emerged as a promising class of biosurfactants for several applications, such as moisturizers, lubricants, and shampoos (Dobler et al., 2016). Rhamnolipids are also effective in the bioremediation of organic and heavy-metal-polluted sites. Consequently, various efforts, including heterologous expression of rhlAB, have been made to establish high-yield rhamnolipid-producing strains. However, P. aeruginosa remains the best rhamnolipid producer. Based on the attenuated strain, further modifications to increase secondary metabolite production would involve the overexpression of proteins that participate in biosynthetic and/or regulatory pathways (Dobler et al., 2016). As expected, the overexpression of rhlAB and estA in wild-type PAO1 and the PAO1aroA mutant strain increased the yield of rhamnolipids (Figure 6B). The rhamnosyltransferase 1 complex (RhlAB) is the key enzyme responsible for transferring the rhamnose moiety to the β-hydroxyalkanoic acid moiety during rhamnolipid biosynthesis (Wang et al., 2007). Rhamnolipid production in P. aeruginosa is regulated by the hierarchical quorum-sensing systems (LasR/R and Rhl/R systems) (Soberón-Chávez et al., 2003). EstA
indirectly influences the synthesis of quorum-sensing molecules by providing the cells with fatty acids (Riedel et al., 2003).

For *P. aeruginosa* PAO1, after electroporation the recombinants were selected on LB agar plates supplemented with gentamycin (15 μg/mL). There were false-positive colonies, which were smaller. According to colony PCR using the internal primer pairs binding to the gentamycin resistance gene, this was not random integration of the cassette (Figure S10). As negative controls we observed that there were gentamycin-resistant colonies of *P. aeruginosa* after electroporation without adding DNA. Most likely the intrinsic multidrug resistance mechanisms of *P. aeruginosa* were enhanced after electroporation as a stress response. However these false-positive colonies were not problematic for screening, as they did not grow in liquid LB containing gentamycin (15 μg/mL) because of antibiotic selection being more stringent in the liquid medium than in the solid medium (data not shown).

Here, we developed recombineering systems for *Pseudomonas* for several reasons including circumventing the inconvenience of constructing a suicide plasmid with long homology arms for genome engineering, which then requires tedious PCR screening of single-crossover and double-crossover events (Hmelo et al., 2015). Further work to construct efficient recombineering systems for more distantly related gram-negative bacteria may require not only the identification of new Red-like or RecET-like operons but also testing combinations of SSBs and Redγ-like proteins with the phage exonuclease/SSAP pairs.

**Limitations of the Study**

We evaluated two *Pseudomonas* endogenous phage recombinant systems (BAS and RecETpsy) in various strains and use the *E. coli* Red and Plu Red recombinant proteins (Redγβα and Pluγβα) as references. These
proteins were expressed using the same inducible promoter and the same plasmid origin. However, we did not assess the expression levels of such proteins, so future experiments are needed to test whether differences in expression levels of these proteins can explain different recombineering efficiencies among strains.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.03.007.

ACKNOWLEDGMENTS
The authors thank Vinoothkannan Ravichandran for critical reading of the manuscript. This study was supported by grants from the National Natural Science Foundation of China (31570094, 31700004, and 31670097), the Shandong Provincial Natural Science Foundation of China (ZR2018ZC2261, ZR2017ZB0212), the 111 Project (B16030), the Science and Technology Development Program of Suzhou (SYG201507), the Natural Science Foundation of Jiangsu Province (BK20160368), the Key Programs of Frontier Scientific Research of the Chinese Academy of Sciences (QYZDY-SSW-SCM008), the State Key Laboratory of Microbial Technology Open Projects Fund (M2017-05), the Huxiang Youth Excellent (2017RS3029) to J.Y., the Recruitment Program of Global Experts in Shandong University to Y.Z., the Qilu Youth Scholar Startup Funding of SDU to H.W., and the Taishan Scholar Program of Shandong Province to J.F. and H.W.

AUTHOR CONTRIBUTIONS
J.Y., Y.Y., A.F.S., Y.Z., and J.F. designed the study. J.Y. and W.Z. performed the informatic analysis, expression plasmid construction, and recombineering efficiency analysis. Y.G. and R.L. performed the mice and C. elegans experiments. C.J., H.W., and A.L. performed rhamnolipid quantification. H.S., X.D., S.L., and H.C. constructed the mutants. J.Y., A.F.S., and J.F. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: October 24, 2018
Revised: December 28, 2018
Accepted: March 7, 2019
Published: April 26, 2019

REFERENCES
Aditi, F.Y., Rahman, S.S., and Hossain, M.M. (2017). A study on the microbiological status of mineral drinking water. Open Microbiol. J. 11, 31–44.

Antoine, R., and Locht, C. (1992). Isolation and molecular characterization of a novel broad-host-range plasmid from Bordetella bronchiseptica with sequence similarities to plasmids from Gram-positive organisms. Mol. Microbiol. 6, 1785–1799.

Aparicio, T., de Lorenzo, V., and Martı´ nez-Garcı´ a, E. (2018). CRISPR/Cas9-based counterselection boosts recombineering efficiency in Pseudomonas putida. Biotechnol. J. 13, 1700161.

Aparicio, T., Jensen, S.I., Nielsen, A.T., de Lorenzo, V., and Martı´ nez-Garcı´ a, E. (2016). The Ssr protein (T1E_1405) from Pseudomonas putida DOT-T1E enables oligonucleotide-based recombineering in platform strain P. putida EM42. Biotechnol. J. 7, 1309–1319.

Beloin, C., Deighan, P., Doyle, M., and Dorman, C.J. (2003). Shigella flexneri 2a strain 2457T expresses three members of the H-NS-like protein family: characterization of the Sfh protein. Mol. Genet. Genom. 270, 66–77.

Biggins, J.B., Kang, H.-S., Ternei, M.A., DeShazer, D., and Brady, S.F. (2014). The chemical arsenal of Burkholderia Pseudomallei is essential for pathogenicity. J. Am. Chem. Soc. 136, 9484–9490.

Bunny, K., Liu, J., and Roth, J. (2002). Phenotypes of lexA mutations in Salmonella enterica: evidence for a lethal lexA null phenotype due to the Fels-2 prophage. J. Bacteriol. 184, 6235–6249.

Carroll, S.F., and Collier, D. (2002). Phenotypes of lexA mutations in Salmonella enterica: evidence for a lethal lexA null phenotype due to the Fels-2 prophage. J. Bacteriol. 184, 6235–6249.

Cook, T.B., Rand, J.M., Nurani, W., Courtney, D.K., Liu, S.A., and Pfleger, B.F. (2018). Genetic tools for reliable gene expression and recombineering in Pseudomonas putida. J. Ind. Microbiol. 45, 517–527.

Court, R., Cook, N., Saikrishnan, K., and Wigley, D. (2007). The crystal structure of lambda-Gam protein suggests a model for RecBCD inhibition. J. Mol. Biol. 371, 25–33.

Derbise, A., Lesic, B., Dacheux, D., Ghigo, J.M., and Carniel, E. (2003). A rapid and simple method for inactivating chromosomal genes in Yersinia. FEMS Microbiol. Lett. 38, 113–116.
Deleber, L., Viela, L.F., Almeida, R.V., and Neves, B.C. (2016). Rhamnolipids in perspective: gene regulatory pathways, metabolic engineering, production and technological forecasting. New Biotechnol. 33, 123–135.

Dong, H., Tao, W., Gong, F., Li, Y., and Zhang, Y. (2013). A functional recT gene for recombineering of ColClamydium. J. Biotechnol. 173, 65–67.

Egan, M., Ramirez, J., Xander, C., Upreti, C., and Bhatt, S. (2016). Lambda red-mediated recombineering in the attaching and effacing pathogen Escherichia albertii. Bioproc. Proced. Online 16, 3.

Egan, S.M., and Schleif, R.F. (1993). A regulatory cascade in the induction of rhaBAD. J. Mol. Biol. 234, 87–96.

Egan, S.M., and Schleif, R.F. (1994). DNA-dependent renaturation of an insoluble DNA binding protein. J. Mol. Biol. 243, 821–829.

Erler, A., Wegmann, S., Elie-Caille, C., Bradshaw, C.R., Maresca, M., Seidel, R., Habermann, B., Muller, D.J., and Stewart, A.F. (2009). Conformational adaptability of RedDJ during DNA annealing and implications for its structural relationship with Rad52. J. Mol. Biol. 391, 586–598.

EUZeBY, J.P. (1997). List of bacterial names with standing in nomenclature: a folder available on the internet. Int. J. Syst. Evol. Microbiol. 47, 590–592.

Fu, J., Bian, X., Hu, S., Wang, H., Huang, F., Seibert, F.M., Plaza, A., Xia, L., Muller, R., Stewart, A.F., et al. (2012). Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat. Biotechnol. 30, 440–446.

Fu, J., Teucher, M., Anastassiadis, K., Skannes, W., and Stewart, A.F. (2010). Chapter eight - a recombineering pipeline to make conditional targeting constructs. In Methods in Enzymology, M.W. Paul and M.S. Philippe, eds. (Academic Press), pp. 125–144.

Gomes, N.C.M., Kosheleva, I.A., Abraham, W.-R., Maresca, M., Seidel, R., Habermann, B., Muller, D.J., and Stewart, A.F. (2010). Chapter eight - a recombineering pipeline to make conditional targeting constructs. In Methods in Enzymology, M.W. Paul and M.S. Philippe, eds. (Academic Press), pp. 125–144.

Han, E.S., Cooper, D.L., Persky, N.S., Suter, V.A., Whitaker, R.D., Montello, M.L., and Lovett, S.T. (2006). RecE exonuclease: substrates, products and interaction with SSB. Nucleic Acids Res. 34, 1084–1091.

Hmeilo, L.R., Barlie, B.R., Almblad, H., Love, M.E., Randall, T.E., Tseng, B.S., Lin, C., Irie, Y., Storek, K.M., Yang, J.J., et al. (2015). Precision-engineering the Pseudomonas aeruginosa genome with two-step allelic exchange. Nat. Protoc. 10, 1820–1841.

Jinno, Y., Chaudhary, V.K., Kondo, T., Adhya, S., Fritzgerald, D.J., and Pastan, I. (1988). Mutational analysis of domain I of Pseudomonas exotoxin. Mutations in domain I of Pseudomonas exotoxin which reduce cell binding and animal toxicity. J. Biol. Chem. 263, 13203–13207.

Kowalczykowski, S.C., and Krupp, B.A. (1987). Effects of Escherichia coli SSB protein on the single-stranded DNA-dependent ATPase activity of Escherichia coli RecA protein. J. Mol. Biol. 193, 97–113.

Latino, L., Esooh, C., Blouin, Y., Tu Thien, H., and Pourcel, C. (2014). A novel Pseudomonas aeruginosa bacteriophage, Ab31, a Chimera formed from temporally Phage PAU2 and P. putida Lytic Phage AF: characteristics and mechanism of bacterial resistance. PLoS One 9, e93777.

Lau, G.W., Hassett, D.J., Ran, H., and Kong, F. (2004). The role of pyocyanin in Pseudomonas aeruginosa infection trends. Mol. Med. 10, 599–606.

Lu, D., Myers, A.R., George, N.P., and Keck, J.L. (2011). Mechanism of exonuclease I stimulation by the single-stranded DNA-binding protein. Nucleic Acids Res. 39, 6536–6545.

Luo, X., Yang, Y., Ling, W., Zhuang, H., Li, Q., and Shang, G. (2017). Production of rhamnolipids by Pseudomonas putida KT2440: markerless gene deletion using a combination of λ Red recombineering and Cre/loxP site-specific recombination. FEMS Microbiol. Lett. 363, fwi014.

Mahajan-Miklos, S., Tan, M.-W., Rahme, L.G., and Ausubel, F.M. (1999). Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa Caenorhabditis elegans pathogenesis model. Cell 96, 47–56.

Maresca, M., Erler, A., Fu, J., Friedrich, A., Zhang, Y., and Stewart, A.F. (2010). Single-stranded heteroduplex intermediates in lambda Red homologous recombination. BMC Mol. Biol. 29, 11–54.

Mathews, D.H., Disney, M.D., Childs, J.L., Schroeder, S.J., Zaker, M., and Turner, D.H. (2004). Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc. Natl. Acad. Sci. U S A 101, 7287–7292.

McEwan, D.L., Kirienko, N.V., and Ausubel, F.M. (2012). Host translational inhibition by Pseudomonas aeruginosa as a candidate intranasal vaccine. Infect. Immun. 70, 1507–1517.

Ricarte, D.E., Martinez-Garcia, E., Nyerges, A., Pál, C., Lorenzo, V., and Aparicio, T. (2018). A standardized workflow for surveying recombinases expands bacterial genome-editing capabilities. Microbiot. Technol. 11, 176–188.

Riedel, K., Talber-Huiber, D., Givskov, M., Schwalb, H., and Eberl, L. (2003). Identification and characterization of a GDSL esterase gene located proximal to the swq quorum-sensing system of Serratia liquefaciens MG1. Appl. Environ. Microbiol. 69, 3901–3910.

Rossi, M.-S., Paquelin, A., Ghigo, J.M., and Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in Serratia marcescens: the inducer and the transported substrate are different molecules. Mol. Microbiol. 48, 1467–1480.

Savoa, D. (2014). New perspectives in the management of Pseudomonas aeruginosa infections. Future Microbiol. 9, 917–928.

Sharan, S.K., Thomason, L.C., Kuznetsova, S.G., and Court, D.L. (2009). Recombineering: a homologous recombination-based method of genetic engineering. Nat. Protoc. 4, 206–223.

Siegel, C.B., Chaudhury, V.K., Fritzgerald, D.J., and Pastan, I. (1989). Functional analysis of domains II, Ib, and III of Pseudomonas exotoxin. J. Biol. Chem. 264, 14256–14261.

Soberón-Chávez, G., Lépine, F., and Déziel, E. (2005). Production of rhamnolipids by...
Pseudomonas aeruginosa. Appl. Environ. Microbiol. 68, 718–725.
Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., et al. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406, 959–964.
Sun, Z., Deng, A., Hu, T., Wu, J., Sun, Q., Bai, H., Zhang, G., and Wen, T. (2015). A high-efficiency recombineering system with PCR-based ssDNA in Bacillus subtilis mediated by the native phage recombinase GP35. Appl. Environ. Microbiol. 99, 5151–5162.
Swingle, B., Bao, Z., Markel, E., Chambers, A., and Cartinhour, S. (2010). Recombineering using RecTE from Pseudomonas syringae. Appl. Environ. Microbiol. 76, 4960–4968.
Szczepańska, A.K. (2009). Bacteriophage-encoded functions engaged in initiation of homologous recombination events. Crit. Rev. Microbiol. 35, 197–220.
Validov, S., Kamilo, F., Qi, S., Wang, J.J., Makarova, N., and Lugtenberg, B. (2007). Selection of bacteria able to control Fusarium oxysporum f. sp. radicis-lycopersici in stonewool substrate. J. Appl. Microbiol. 102, 461–471.
Van Kessel, J.C., and Hatfull, G.F. (2006). Recombineering in Mycobacterium tuberculosis. Nat. Methods 4, 147–152.
Wang, J., Sarov, M., Rientjes, J., Fu, J., Hollak, H., Kranz, H., Kie, W., Stewart, A.F., and Zhang, Y. (2006). An improved recombineering approach by adding RecA to lambda Red recombination. Mol. Biotechnol. 32, 43–53.
Wang, Q., Fang, X., Bai, B., Liang, X., Shuler, P.J., Goddard, W.A., and Tang, Y. (2007). Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. Biotechnol. Bioeng. 98, 842–853.
Wilhelm, S., Gdynia, A., Tielen, P., Rosenau, F., and Jaeger, K.E. (2007). The autotransporter esterase EstA of Pseudomonas aeruginosa is required for rhamnolipid production, cell motility, and biofilm formation. J. Bacteriol. 189, 6695–6703.
Xin, X.-F., and He, S.Y. (2013). Pseudomonas syringae pv. tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. Annu. Rev. Phytopathol. 51, 473–498.
Yang, P., Wang, J., and Qi, Q. (2015). Prophage recombinases-mediated genome engineering in Lactobacillus plantarum. Microb. Cell Fact. 14, 1–11.
Yang, S.H., Zhou, R., Campbell, J., Chen, J., Ha, T., and Paull, T.T. (2013). The SCSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1. EMBO J. 32, 126–139.
Yang, Z.-S., Ma, L.-Q., Zhu, K., Yan, J.-Y., Bian, L., Zhang, K.-Q., and Zou, C.-G. (2016). Pseudomonas toxin pyocyanin triggers autophagy: implications for pathoadaptive mutations. Autophagy 12, 1015–1028.
Yin, J., Zhu, H., Xia, L., Ding, X., Hoffmann, T., Hoffmann, M., Bian, X., Müller, R., Fu, J., Stewart, A.F., et al. (2015). A new recombineering system for Photorhabdus and Xenorhabdus. Nucleic Acids Res. 43, e36.
Zhang, L., Veres-Schallnat, T.A., Somogyi, A., Pemberton, J.E., and Maier, R.M. (2012). Fatty acid cosubstrates provide β-Oxidation precursors for rhamnolipid biosynthesis in Pseudomonas aeruginosa, as evidenced by isotope tracing and gene expression assays. Appl. Environ. Microbiol. 78, 8611–8622.
Zhang, Q., Xiao, Q., Xu, J., Tong, Y., Wen, J., Chen, X., and Wei, L. (2015). Effect of retS gene on antibiotics production in Pseudomonas fluorescens FD6. Microbiol. Res. 180, 23–29.
Zhang, Y., Buchholz, F., Muyrers, J.P.P., and Stewart, A.F. (1998). A new logic for DNA engineering using recombination in Escherichia coli. Nat. Genet. 20, 123–128.
Zhang, Y., Muyrers, J.P.P., Testa, G., and Stewart, A.F. (2000). DNA cloning by homologous recombination in Escherichia coli. Nat. Biotechnol. 18, 1314–1317.
Zhou, F., Cui, Q., Han, S., Dong, H., Zhang, J., Ma, F., and Zhang, Y. (2015). Enhanced rhamnolipid production of Pseudomonas aeruginosa SG by increasing copy number of rhlAB genes with modified promoter. RSC Adv. 5, 70546–70552.
Supplemental Information

Single-Stranded DNA-Binding Protein and Exogenous RecBCD Inhibitors Enhance Phage-Derived Homologous Recombination in *Pseudomonas*

Jia Yin, Wentao Zheng, Yunsheng Gao, Chanjuan Jiang, Hongbo Shi, Xiaotong Diao, Shanshan Li, Hanna Chen, Hailong Wang, Ruijuan Li, Aiying Li, Liqiu Xia, Yulong Yin, A. Francis Stewart, Youming Zhang, and Jun Fu
Supplementary information

TRANSPARENT METHOD

Strains, plasmids and reagents

The bacterial strains and plasmids used are listed in Table S1 and Table S2. The expression plasmids used to evaluate homologous recombination efficiencies are based on the pBBR1 origin (Antoine and Locht, 1992) and the \( \text{rhaR-rhaS} \) \( \text{P}_{\text{Rha}} \) promoter (Egan and Schleif, 1993, 1994). The plasmids were constructed by recombineering either in GB08-red or in GB05-dir (Fu et al., 2012; Fu et al., 2010). Oligonucleotides were synthesized by Sangon Biotech (Shanghai) Co., Ltd., in China (Table S3). Phusion polymerase, restriction enzymes and DNA markers were purchased from New England Biolabs (Massachusetts, USA). Antibiotics were supplied by Invitrogen (Massachusetts, USA). *E. coli*, *P. aeruginosa*, *P. fluorescens* and *P. putida* were cultured in Luria-Bertani (LB) broth or on LB agar plates (1.2% agar) (Bertani, 1951); *P. syringae* was cultured in Kings B medium (King et al., 1954). The concentrations of the required antibiotics are listed in Table S4.

Preparation of expression plasmid

The expression plasmids used to evaluate homologous recombination efficiencies are based on the pBBR1 origin (Antoine and Locht, 1992) and the \( \text{rhaR-rhaS} \) \( \text{P}_{\text{Rha}} \) promoter (Egan and Schleif, 1993, 1994), and constructed by four steps. First, pGDFG10 (unpublished in our lab), named pBBR1-Rha-\( \text{TE}_{\text{Psy}} \)-kan in the present study, was digested by XmnI, and the restriction fragment was purified by agarose gel. Second, \( \text{red}_{\beta} \) and \( \text{plu}_{\beta} \) were PCR amplified from pSC101-BAD-\( \text{red}_{\beta} \)-\( \text{A}_{4} \)-tet (Fu et al., 2010) and pSC101-BAD-\( \text{plu}_{\beta} \)-amp (Yin et al., 2015), respectively. The function genes of BAS were synthesised from GENEWIZ (Suzhou) Co., Ltd., in China. Third, the restriction fragment and function genes were co-transformation in GB05-dir (Fu et al., 2012) by recombineering to construct pBBR1-Rha-\( \text{red}_{\beta} \)-Kan, pBBR1-Rha-\( \text{plu}_{\beta} \)-Kan and pBBR1-Rha-BAS-Kan. Fourth, the \( \text{red}_{\gamma} \) and \( \text{plu}_{\gamma} \) was inserted into pBBR1-Rha-\( \text{TE}_{\text{Psy}} \)-kan and pBBR1-Rha-BAS-kan by recombineering in GB08-red (Fu et al., 2010).
Electrocompetent cell preparation and recombineering

Various expression plasmids were electroporated into *E. coli* GB2005, *P. aeruginosa* PAO1, *P. fluorescens* pf5, *P. syringae* DC3000, and *P. putida* KT2440. *E. coli* electrocompetent cells were prepared as reported previously (Fu et al., 2010). For *P. aeruginosa, P. fluorescens, P. syringae* and *P. putida*, overnight cultures containing the expression plasmids were diluted into 1.3 mL of LB medium with the appropriate antibiotics. The starting OD$_{600}$ values were approximately 0.085. Fresh cultures were grown at 30°C or 37°C according to the optimum growth temperature for each strain and at 950 rpm for an appropriate amount of time until the log phase began for each strain. After the addition of the inducer L-rhamnose to a final concentration of 2.5 mg mL$^{-1}$, the cells were grown at 30°C or 37°C and 950 rpm for 40 min. Cells were then centrifuged for 30 s at 9500 rpm at room temperature. The supernatants were discarded, and the cell pellets were resuspended in 1 mL of ddH$_2$O and centrifuged. The washing procedure was repeated one more time. Then, cells were resuspended in 30 µL of ddH$_2$O, and DNA was added. For the experiments shown in Figure 3B-H and Figure 4B and C, 1.5 µg of PCR product was used. Two to five micrograms of PCR product were used for the genome engineering of *P. aeruginosa, P. fluorescens* and *P. syringae*. Electroporation was performed using 1-mm cuvettes and an Eppendorf 2510 electroporator set at 1250 V. One millilitre of the appropriate medium was added after electroporation. The cells were incubated at 30°C for 100 min or 37°C for 60 min with shaking and then spread on plates containing the appropriate antibiotics.

*P. aeruginosa* virulence assays in BALB/c mice and *Caenorhabditis elegans* (*C. elegans*)

Wild-type *P. aeruginosa* PAO1 and the PAO1aroA mutant (1*10^9 CFU) were injected intraperitoneally into the mice. Mice were observed for mortality for a period of 7 days. For the *C. elegans* assay, the wild-type PAO1 strain and PABMT mutants (1*10^8 CFU) were spread on Nematode Growth Medium (NGM) (3 g L$^{-1}$ NaCl, 17 g L$^{-1}$ agar, 2.5 g
L^-1 peptone, 5 mg L^-1 cholesterol, 1 mM MgSO_4, 1 mM CaCl_2, 2.7 g L^-1 KH_2PO_4, 0.9 g L^-1 K_2HPO_4, pH 6.0) plates. Plates were incubated at 37°C for 18-24 h and then placed at room temperature for 8-12 h. Fifteen to twenty L4 stage C. elegans were placed on the assay plate, which was then incubated at 16°C. Each independent assay consisted of three replicates. Worm phenotypes were observed by microscopy after 5 days.

**Rhamnolipid quantification**

Wild-type P. aeruginosa PA01 and mutant strains were first grown in LB broth with 10 µg mL^-1 gentamycin for 24 h at 37°C with shaking, then diluted 1:10 into mineral salt medium(Wang et al., 2007) supplemented with aromatic amino acids without antibiotics and finally incubated for 2 days.

Rhamnolipid quantification was based on a previously described method(Wang et al., 2007) with slight modifications. Cells were centrifuged for 60 s at 13200 rpm after fermentation, and 1 mL of the supernatant was then extracted with 1 mL of ethyl ether. The organic phase was evaporated to dryness, and 0.1 mL of ddH_2O was added. The sample was diluted 1:10 in a solution containing 0.19% orcinol in 53% H_2SO_4. After heating for 30 min at 80°C, the samples were cooled at room temperature, and the OD_421 was measured(Koch et al., 1991). The rhamnolipid concentration was calculated using a standard curve for L-rhamnose (0-50 mg L^-1) and expressed in triplicate as the rhamnose concentration.

**Animals**

Male BALB/c mice were provided by the School of Medicine of Shandong University. All male mice were housed under specific pathogen-free conditions within the animal facility. Mice were 6-8 weeks old at the start of the experiments.

**Statistical analyses**

All data were analysed by two-tailed Student’s t-test and were conducted in Prism. A P-value that is less than 0.05 is considered statistically significant.

**Ethics statement**
All mice experiments in this study were approved and supervised by the Institutional Animal Care and Use Committee of Animal Laboratory Center of Shandong University.
Table S1. Bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas putida*, related to Figure 2, 3, 4 and 5.

| Strain               | Genome type                                                                                                                                                                                                 | Optimum growth temperature (°C) | References or sources      |
|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-----------------------------|
| GB2005               | F-mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ. rpsL. nupG θfluA::IS2 recET reda, phage T1-resistant (E. coli) | 37                            | (Fu, 2010)                  |
| GB05-dir             | GB2005, araC-BAD-ETγA (E. coli)                                                                                                                                                                             | 37                            | (Fu et al., 2012)           |
| GB08-red             | GB2005, araC-BAD-γβA, ΔlacZ (E. coli)                                                                                                                                                                         | 37                            | (Fu, 2010)                  |
| *P. aeruginosa* PA01 | Wild-type strain of *Pseudomonas aeruginosa*                                                                                                                                                                | 37                            | (Stover et al., 2000)       |
| PA01aroA             | *Pseudomonas aeruginosa* PA01,  ΔaroA                                                                                                                                                                       | 37                            | This study                  |
| PABMT                | *Pseudomonas aeruginosa* PA01, ΔphnAB, ΔlasB, ΔmdoH and toxA<sub>ET371D</sub>                                                                                                                             | 37                            | This study                  |
| *P. fluorescens* pf5 | Wild-type strain of *Pseudomonas fluorescens* pf5                                                                                                                                                           | 30                            | (Paulsen et al., 2005)      |
| PF5retS              | *Pseudomonas fluorescens* pf5, ΔretS                                                                                                                                                                        | 30                            | This study                  |
| *P. syringae* DC3000 | Wild-type strain of *Pseudomonas syringae pv. tomato str. DC3000                                                                                                                                              | 30                            | (Buell et al., 2003)        |
| *P. syringae* BGC6   | P<sub>ama</sub> insertion for expression of gene cluster BGC6 in *Pseudomonas syringae pv. tomato str. DC3000, GentaR                                                                                      | 30                            | This study                  |
| *P. putida* KT2440   | Wild-type strain of *Pseudomonas putida* KT2440                                                                                                                                                              | 30                            | (Gross et al., 2006)        |
Table S2. Expression plasmids and PCR template, related to Figure 3 and 4.

| Plasmid                          | Characteristics                                      | References or sources |
|----------------------------------|------------------------------------------------------|-----------------------|
| pBBR1-Rha-redγβα-Kan             | redγβα under Rha promoter                            | This study            |
| pBBR1-Rha-pluγβα-kan             | pluγβα under Rha promoter                            | This study            |
| pBBR1-Rha-TE_{Psy}-kan           | recTE_{Psy} under Rha promoter                       | This study            |
| pBBR1-Rha-pluγTE_{Psy}-kan       | recTE_{Psy} and pluγ under Rha promoter              | This study            |
| pBBR1-Rha-redγTE_{Psy}-kan       | recTE_{Psy} and redγ under Rha promoter              | This study            |
| pBBR1-Rha-BAS-kan                | BAS gene under Rha promoter                          | This study            |
| pBBR1-Rha-pluγBAS-kan            | BAS gene and pluγ under Rha promoter                 | This study            |
| pBBR1-Rha-redγBAS-kan            | BAS gene and redγ under Rha promoter                 | This study            |
| pRK2-apra-cm                     | PCR templates to amplify apraR gene                  | This study            |
| pEX18Gm                          | PCR templates to amplify sacB-gentaR gene            | (Ni et al., 2008)     |
| pR6K-Tps-gentaR-tetR-T7RP        | PCR templates to amplify lox71-gentaR-lox66 or gentaR gene | (Bian et al., 2012)   |
| pSC101-BAD-ETg-tet               | recET and redγ under BAD promoter                    | (Fu et al., 2012)     |
| pSC101-BAD-ETgSSB_{E.coli}~tet   | recET, redγ and SSB from E. coli genome under BAD promoter | This study            |
| pSC101-BAD-ETgSSB_{F.plasmid}~tet | recET, redγ and SSB from E. coli F plasmid under BAD promoter | This study            |
| Gene          | Application                  | Primers 1 (5’ - 3’)                                                                 | Primers 2 (5’ - 3’)                                                                 |
|--------------|------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| playTE       | Expression plasmid           | ACATGCAAATTTTGATCGTGACGGACGGACATATAGATGGGACATCTTGCCTCTTATATATCTCCCA TTTTAC | GCCGCTTTTATAGACGGGCGTCGTAATGGAACACCTTACCTAAGAAGGAGATATACATGGAACCCCATATGCAGTTATG |
| redγTE       | Expression plasmid           | ACATGCAAATTTTGATCGTGACGGACGGACATATAGATGGGACATCTTGCCTCTTATATATCTCCCA TTTTAC | GCCGCTTTTATAGACGGGCGTCGTAATGGAACACCTTACCTAAGAAGGAGATATACATGGAACCCCATATGCAGTTATG |
| playBAS      | Expression plasmid           | TCGTAGCGGTGGCCCATGCAGGAGCGGTGTTATGAGCAGGATCGATACATCAATGCAGTTTAC | GCCGCTTTTATAGACGGGCGTCGTAATGGAACACCTTACCTAAGAAGGAGATATACATGGAACCCCATATGCAGTTATG |
| redγBAS      | Expression plasmid           | TCGTAGCGGTGGCCCATGCAGGAGCGGTGTTATGAGCAGGATCGATACATCAATGCAGTTTAC | GCCGCTTTTATAGACGGGCGTCGTAATGGAACACCTTACCTAAGAAGGAGATATACATGGAACCCCATATGCAGTTATG |
| aroA-genta- | AroA Knock-out               | CAAGTCGATTTCCCATCGCTCGATCATGCTCGGCTCCCTGGCCGAAGGCACCACCGAAGTGGAGGGCTTCCTCGA | CAATTGGGGGAAAAGGGGGGTTACCGATGATGAACATGCCGATACATCGGCGTGGCCATCGTGCATCCGGACACAGGACAGGCAGGAGTGGAGGGCTTCCTCGA |
| loxM-3       |                               | CAATTGGGGGAAAAGGGGGGTTACCGATGATGAACATGCCGATACATCGGCGTGGCCATCGTGCATCCGGACACAGGACAGGAGTGGAGGGCTTCCTCGA | CAATTGGGGGAAAAGGGGGGTTACCGATGATGAACATGCCGATACATCGGCGTGGCCATCGTGCATCCGGACACAGGACAGGAGTGGAGGGCTTCCTCGA |
| AB-genta-    | phnAB Knock-out              | TTCTCCACGACAGCCTCGGGCGTGTTGAGGAATCGACTCGGGATGGAACATGCAAGCCACGCTTGCGATTGCGC | TTCTCCACGACAGCCTCGGGCGTGTTGAGGAATCGACTCGGGATGGAACATGCAAGCCACGCTTGCGATTGCGC |
| LoxM-3       |                               | CAATTGGGGGAAAAGGGGGGTTACCGATGATGAACATGCCGATACATCGGCGTGGCCATCGTGCATCCGGACACAGGACAGGAGTGGAGGGCTTCCTCGA | CAATTGGGGGAAAAGGGGGGTTACCGATGATGAACATGCCGATACATCGGCGTGGCCATCGTGCATCCGGACACAGGACAGGAGTGGAGGGCTTCCTCGA |
| lasB-genta-  | lasB Knock-out               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| loxM-3       |                               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| lasB-genta-  |                               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| loxM-5       |                               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| lasB-genta-  |                               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| loxM-5       |                               | GAAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT | GAGGCGCTTCTCGGGGATACCCACGCGCGCAATGGCCAACAGGATAGCGAGCCGCTGGAGGT |
| Gene  | Homology Arms (Underlined) | mdoH Knock-out | retS Knock-out | tox point mutation | Insertion promoter |
|-------|-----------------------------|-----------------|----------------|--------------------|------------------|
| *mdoH*-genta-loxM | CCTCTCCCCAGACCCCGGTATGCAGCCGAAAGTGCTACCGGGTCACCTACACGAGCCACAACTTTAATGTGAAAGTGGGTC | **mdoH** Knock-out | TGGAGCATGTTGAGGAGGTCACGAGAAGGAGGCGAGGAGAGATTTAACAGGCACAGCCGAGGCTCAGGCTCAAAACTTTAATGTGAAAGTGGGTC | GCACACGCCCCTTGGCGTGCAGTAGTTGAGAAGCCGACAAACGAGGGATTTCGCCGA | **Insertion promoter** |
| *retS*-genta-loxM | TGGAGCATGTTGAGGAGGTCACGAGAAGGAGGCGAGGAGAGATTTAACAGGCACAGCCGAGGCTCAGGCTCAAAACTTTAATGTGAAAGTGGGTC | **retS** Knock-out | GCACACGCCCCTTGGCGTGCAGTAGTTGAGAAGCCGACAAACGAGGGATTTCGCCGA | GCACACGCCCCTTGGCGTGCAGTAGTTGAGAAGCCGACAAACGAGGGATTTCGCCGA | **Insertion promoter** |
| *toxA*-sacB-genta | GCCGACGTTGCGCGGTGGAGGTCACGAGAAGGAGGCGAGGAGAGATTTAACAGGCACAGCCGAGGCTCAGGCTCAAAACTTTAATGTGAAAGTGGGTC | **toxA** point mutation | TGGAGCATGTTGAGGAGGTCACGAGAAGGAGGCGAGGAGAGATTTAACAGGCACAGCCGAGGCTCAGGCTCAAAACTTTAATGTGAAAGTGGGTC | GCACACGCCCCTTGGCGTGCAGTAGTTGAGAAGCCGACAAACGAGGGATTTCGCCGA | **Insertion promoter** |
| *BGC6*-apra | | **Insertion promoter** | | | **Insertion promoter** |

The homology arms are underlined.

**Verify the plasmid recombineering (Figure 3)**

**Verify the genome engineering of mdoH**

**PCR product in the GentaR sequence**
Table S4. Antibiotic concentrations used in different strains, related to Figure 3.

| Strain                                             | Antibiotic (µg/ml) | Kanamycin [km] | Gentamycin [genta] | Apramycin [apra] | Tetracycline [tet] | Ampicillin [amp] | Chloramphenicol [cm] |
|----------------------------------------------------|--------------------|----------------|--------------------|-------------------|-------------------|-----------------|----------------------|
| *E. coli*                                          | 15                 | 5              | 30                 | 5                 | 100               | 15              |                      |
| *P. aeruginosa* PAO1                               | 300                | 15             | 60                 | 50                | ND\(^b\)          | 90              |                      |
| *P. fluorescens* pf5                               | 10                 | 15             | 20                 | 35                | ND                | ND              |                      |
| *P. putida* KT2440                                  | 15                 | 10             | 40                 | --\(^a\)          | 300               | ND              |                      |
| *P. syringae* pv. tomato str. DC3000                | 15                 | 3              | 20                 | 3                 | 200               | ND              |                      |

\(^a\)Did not test this antibiotic

\(^b\)Can not use this antibiotic
Table S5. RecBCD sequence alignment between *Pseudomonas* and *E. coli/P. luminescens*, related to Figure 1.

|        | Identities | Positives | Gaps   | Identities | Positives | Gaps   |
|--------|------------|-----------|--------|------------|-----------|--------|
| *P. aeruginosa* |             |           |        |            |           |        |
| RecB   | 499/1254 (40%) | 680/1254 (54%) | 109/1254 (8%) | 506/1259 (40%) | 678/1259 (53%) | 92/1259 (7%) |
| RecC   | 448/1160 (39%) | 619/1160 (53%) | 125/1160 (10%) | 422/1181 (36%) | 604/1181 (51%) | 129/1181 (10%) |
| RecD   | 287/670 (43%) | 364/670 (54%) | 74/670 (11%) | 287/670 (43%) | 364/670 (54%) | 74/670 (11%) |
| *P. fluorescens* |             |           |        |            |           |        |
| RecB   | 513/1248 (41%) | 691/1248 (55%) | 127/1248 (10%) | 489/1248 (39%) | 675/1248 (54%) | 96/1248 (7%) |
| RecC   | 443/1112 (40%) | 605/1112 (54%) | 118/1112 (10%) | 433/1168 (37%) | 608/1168 (52%) | 134/1168 (11%) |
| RecD   | 281/659 (43%) | 367/659 (55%) | 75/659 (11%) | 281/659 (43%) | 367/659 (55%) | 75/659 (11%) |
| *P. putida* |             |           |        |            |           |        |
| RecB   | 493/1217 (41%) | 675/1217 (55%) | 69/1217 (5%) | 488/1235 (40%) | 677/1235 (54%) | 86/1235 (6%) |
| RecC   | 451/1105 (41%) | 613/1105 (55%) | 94/1105 (8%) | 424/1113 (38%) | 587/1113 (52%) | 99/1113 (8%) |
| RecD   | 291/660 (44%) | 384/660 (58%) | 70/660 (10%) | 291/660 (44%) | 384/660 (58%) | 70/660 (10%) |
| *P. syringae* |             |           |        |            |           |        |
| RecB   | 512/1242 (41%) | 693/1242 (55%) | 105/1242 (8%) | 486/1236 (39%) | 673/1236 (54%) | 80/1236 (6%) |
| RecC   | 444/1145 (39%) | 619/1145 (54%) | 125/1145 (10%) | 426/1164 (37%) | 602/1164 (51%) | 137/1164 (11%) |
| RecD   | 277/662 (42%) | 371/662 (56%) | 78/662 (11%) | 344/669 (56%) | 427/669 (70%) | 10/669 (1%) |

Results from NCBI blastp (protein-protein BLAST) using default parameters.
Table S6. Induction and preparation of electrocompetent cells for four *Pseudomonas* strains, related to Figure 2 and 3.

|                  | *P. aeruginosa* | *P. putida* | *P. syringae* | *P. fluorescens* |
|------------------|-----------------|-------------|---------------|------------------|
| Induction        | Time            | 2h          | 2h            | 3h               | 1h               |
|                  | OD<sub>600</sub> | 0.30        | 0.80          | 0.70             | 0.15             |
| Electrocompetent cells preparation | Time | 2.5h | 2.5h | 4h | 1.5h |
|                  | OD<sub>600</sub> | 0.60        | 1.50          | 1.10             | 0.35             |

* The overnight culture was diluted to OD<sub>600</sub> ≈ 0.085 as the starting culture.
Table S7. Pathogenicity of *P. aeruginosa* in mice, related to Figure 5.

|           | LD50 (*10^8 CFU)* | 95% confidence intervals (*10^8 CFU)* |
|-----------|-------------------|---------------------------------------|
| Wild type | 4.141             | 3.432-4.850                           |
| PABMTb    | 4.339             | 3.552-5.126                           |

*a*. Mortalities were recorded after 12 h, and 50% lethal dose (LD50) values with 95% confidence intervals were determined using SPSS analysis. These results were determined from three independent assays, and five concentrations for each concentration-response experiment were used.

*b*. PABMT is a *P. aeruginosa* mutant (*phnAB*, *lasB* *mdoH* and *toxA*).
Figure S1. Protein sequence alignments between Redβ (beta) and orf38(A), Redα (alpha) and orf37 (B), and single-strand DNA-binding protein (SSB) and orf36 (C). Conserved sequences are shaded in black, Related to Figure 1.
Figure S2. Optimization of transformation in Pseudomonas, Related to Figure 2. (A) and (C) Growth curves for P. fluorescens and P. putida. The optical density at 600 nm (OD$_{600}$) was measured from a starting OD$_{600}$ of 0.085. (B) and (D) Cell competence time windows for P. fluorescens and P. putida. The plasmid pBBR1-Rha-GFP-km was electroporated into Pseudomonas, and colonies were selected on kanamycin plates and counted. Data represent the mean ± SD from three independent experiments. The statistical analysis used is Student’s t test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure S3. Optimization of recombineering in *Pseudomonas* species using the assay shown in Fig. 3A, Related to Figure 2 and 3. Titration of the DNA amount and comparison of the homology in *P. fluorescens* harbouring the RedγTEPsy expression plasmid (A) and (B), in *P. syringae* harbouring the BAS expression plasmid (C) and (D) and in *P. putida* harbouring the BAS expression plasmid (E) and (F), in *P. aeruginosa* harbouring the PluγTEPsy expression plasmid (G) and (H). Colonies were selected on gentamycin plates and counted. Data represent the mean ± SD from three independent experiments. The statistical analysis used is Student’s *t* test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure S4. PCR verification of point mutations via SacB counterselection in *P. aeruginosa*, Related to Figure 5. (A) Diagram of seamless genome engineering in *P. aeruginosa* and schematic presentation of the PCR setup for verification. (B) Insertion of the SacB-genta cassette into the *toxA* target. Lane C is the wild-type strain, used as a negative control. Lane M is the Takara DL5000 marker. Lane 1 and 2 are recombinants. Both clones 1 and 2 are correct. (C) Introduction of the point mutation via oligo repair. The three clones are correct. (D) Sequencing verification of the mutant.
Figure S5. SacB function test on an LB plate containing 10% sucrose (left panel) and an LB plate without sucrose (right panel), Related to Figure 5. 1, Wild-type *P. aeruginosa* PAO1; 2-8, different PAO1 mutant colonies after SacB-genta cassette insertion.
Figure S6. Colony PCR verification for genome engineering of *P. syringae pv. tomato* str. DC3000 and *P. fluorescens* pf5. PCRs were set up across the intact insertion, Related to Figure 5. (A) Insertion of the P\textsubscript{genta} promoter for BGC6 expression in *P. syringae pv. tomato* str. DC3000. All three clones are correct. (B) Gene knockout of *retS* in *P. fluorescens* pf5. Lane 1 is the mutant strain containing the gentaR-loxM cassette (Pf5-ΔretS-genta-loxM); the clone is correct. Lanes 2 and 3 are the final mutant strain after Cre site-specific recombination (Pf5-ΔretS).
Figure S7. Toxicity test for wild-type PAO1 and the PABMT mutant (1*10^8 CFU) using C. elegans. Scale bar, 1mm, Related to Figure 5.
Figure S8. Growth curves of wild-type PAO1 and the PAO1aroA mutant, Related to Figure 6. The optical density at 600 nm (OD$_{600}$) was measured per hour from a starting OD$_{600}$ of 0.085. Data represent the mean ± SD from three independent experiments.
Figure S9. Diagram of the expression plasmid pBBR1-EstA-genta-RhlAB, Related to Figure 6.
Figure S10. PCR verification of the false positive clones of *P. aeruginosa* from RedγBAS recombineering at 37°C, Related to Figure 3. (A) Diagram of the genome/plasmid engineering and schematic presentation of the PCR setup. PCR using primers (red arrows, a1/a2) across the insertion site was set up to distinguish the wild type and the recombinants. PCR using internal primer pairs (green arrows, b1/b2) binding to the gentamycin resistance gene (GentaR) was set up to check if there was any random integration of the cassette. (B) PCR verification of the plasmid engineering from the assay in Figure 3A. Lane M is the Takara DL5000 marker; Lane 1: correct clone using primers Pchk-a1/a2 (Table S3) for PCR product of 1167bp; Lane 2-10: false positive clones using primers Pchk-a1/a2 for PCR product of 4662 bp, which can not be amplified for the given polymerase and the PCR conditions; Lane 11-12: false positive clones from negative control without DNA electroporation, using primers Pchk-a1/a2 for PCR; Lane 13: correct clone using primers Gent-b1/b2 (Table S3) for PCR product of 534bp; Lane 14-22: false positive clones using primers Gent-b1/b2 for PCR product of 534bp if there was any random integration of the cassette; Lane 23-24: false positive clones from negative control without DNA electroporation, using primers Gent-b1/b2 for PCR. (C) PCR verification of the genome engineering (*mdoH* knocking out). Lane 1: correct clone using primers Hchk-a1/a2 (Table S3) for PCR product of 1299bp; Lane 2-10: false positive clones using primers Hchk-a1/a2 for PCR product of 2872 bp; Lane 11-12: false positive clones from negative control without DNA electroporation, using primers Hchk-a1/a2 for PCR; Lane 13: correct clone using primers Gent-b1/b2 for PCR product of 534bp; Lane 14-22: false positive clones using primers Gent-b1/b2 for PCR product of 534bp if there was any random integration of the cassette; Lane 23-24: false positive clones from negative control without DNA electroporation, using primers Gent-b1/b2 for PCR.
References:
Bian, X., Huang, F., Stewart, F.A., Xia, L., Zhang, Y., and Müller, R. (2012). Direct Cloning, Genetic Engineering, and Heterologous Expression of the Syringolin Biosynthetic Gene Cluster in E. coli through Red/ET Recombineering. ChemBioChem 13, 1946-1952.
Buell, C., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I., Gwinn, M., Dodson, R., Deboy, R., Durkin, A., and Kolonay, J. (2003). The complete genome sequence of the Arabidopsis and tomato pathogen Pseudomonas syringae pv. tomato DC3000. Proc Natl Acad Sci USA 100, 10181 - 10186.
Fu, J., Bian, X., Hu, S., Wang, H., Huang, F., Seibert, P.M., Plaza, A., Xia, L., Müller, R., Stewart, A.F., et al. (2012). Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat Biotechnol 30, 440-446.
Fu, J., Teucher, M., Anastassiadis, K., Skarnes, W., Stewart, A.F. (2010). A Recombineering Pipeline to Make Conditional Targeting Constructs. Methods Enzymol 477, 125-144.
Gross, F., Ring, M.W., Perlova, O., Fu, J., Schneider, S., Gerth, K., Kuhlmann, S., Stewart, A.F., Zhang, Y., and Muller, R. (2006). Metabolic engineering of Pseudomonas putida for methylmalonyl-CoA biosynthesis to enable complex heterologous secondary metabolite formation. Chem Biol 13, 1253-1264.
Ni, B., Du, Z., Guo, Z., Zhang, Y.M., and Yang, R.F. (2008). Curing of four different plasmids in Yersinia pestis using plasmid incompatibility. Letters in Applied Microbiology 47, 235-240.
Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., DeBoy, R.T., Seshadri, R., Ren, Q., Madupu, R., et al. (2005). Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5. Nat Biotech 23, 873-878.
Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., et al. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406, 959-964.