Genome modifications and cloning using a conjugally transferable recombineering system

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A B S T R A C T

The genetic modification of primary bacterial disease isolates is challenging due to the lack of highly efficient genetic tools. Herein we describe the development of a modified PCR-based, λ Red-mediated recombineering system for efficient deletion of genes in Gram-negative bacteria. A series of conjugally transferrable plasmids were constructed by cloning an *oriT* sequence and different antibiotic resistance genes into recombinogenic plasmid pKD46. Using this system we deleted ten different genes from the genomes of *Edwardsiella ictaluri* and *Aeromonas hydrophila*. A temperature sensitive and conjugally transferable *flp* recombinase plasmid was developed to generate markerless gene deletion mutants. We also developed an efficient cloning system to capture larger bacterial genetic elements and clone them into a conjugally transferrable plasmid for facile transferring to Gram-negative bacteria. This system should be applicable in diverse Gram-negative bacteria to modify and complement genomic elements in bacteria that cannot be manipulated using available genetic tools.

1. Introduction

Genetic manipulation of bacterial strains provides critical information on the contributions of specific loci to virulence or other cellular functions, and many systems have been developed to achieve genetic knockouts and modifications [4,5,18]. The modification of bacterial genomes using counter-selectable double-crossover methods are labor intensive and sometimes very difficult to achieve due to the low frequency of recombination events [21,26,31]. In contrast, the λ Red recombineering system [39,41] has many advantages as a fast, efficient and reliable means of generating targeted genetic modifications in prokaryotes [11,61] and eukaryotes [7]. The λ Red system expresses Exo, Beta and Gam proteins that work coordinately to recombine single and double stranded DNA [11,38,61], and has been exploited for genome modifications in *Escherichia coli*, *Salmonella enterica* and other Gram-negative bacteria [9,11,40,61]. Exo has a 5′–3′ double stranded DNA (dsDNA)-dependent exonuclease activity for generating 3′ single stranded DNA (ssDNA) overhangs [6,32,34] which then serve as a substrate for ssDNA-binding protein Beta to anneal complementary DNA strands for recombination [8,28,38]. Gam, an inhibitor of host exonuclease activity due to RecBCD [44], helps to improve the efficiency of λ Red-mediated recombination with linear double-strand DNA. Unlike *recA*-dependent homologous recombination which requires longer regions of sequence homology with the targeted genetic region [25], the λ Red apparatus can efficiently recombine DNA with homologous regions as short as 30–50 bp which can directly be incorporated into oligonucleotide primers in a PCR [11,61]. The recombineering technique is widely used to generate precise deletions [11], substitutions [33], insertions [36] or tagging [57] of targeted genes. One of the biggest advantages of the recombineering method is that modifying DNA can precisely eliminate the antibiotic selection markers for subsequent modification of the targeted DNA [11,42,67].

While this recombineering system works well in a model bacterium such as *E. coli* [37,39], bacteria often express restriction endonucleases that make them recalcitrant to foreign DNA even among naturally competent strains [1,3]. In fact, it was the study of experimental infections of *E. coli* strains with bacteriophage λ that led to the discovery of restriction-modification (RM) systems [2]. Overcoming host RM systems can be accomplished via the passage of plasmids through a methylation-minus *E. coli* strain [51], but in highly methylated bacterial strains it may be necessary to use an *in vitro* or *in vivo* methylation strategy to achieve more efficient electroporation [12,13,29]. However, modulating the plasmid DNA

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methylation status is inefficient and labor-intensive compared to using conjugal transfer to introduce foreign DNA into a bacterial strain using a broad host range plasmid like IncP when electroporation is problematic [14,15,17].

Our need to generate targeted genetic deletions in Gram-negative bacterial pathogens of farmed catfish led to the development of recombinogenic plasmids that could be introduced into Gram-negative bacteria via conjugation. Our studies focused on two bacterial pathogens, including motile *Aeromonas* septicemia (MAS) and enteric septicemia of catfish (ESC) caused by *Aeromonas hydrophila* and *Edwardsiella ictaluri*, respectively, which are responsible for significant economic losses to the channel catfish industry in the Southeastern United States [56]. Fish diseases caused by strains of *E. ictaluri* are also frequently reported in catfish farming in Asia [46]. While *E. ictaluri* was formerly the most important bacterial pathogen in farmed US catfish, in 2009 US catfish farmers experienced epidemic disease outbreaks of motile *Aeromonas* septicemia (MAS) caused by a highly virulent

Aeromonas hydrophila strain [20]. This newly emergent and virulent *A. hydrophila* strain, which has been implicated to have an Asian origin [23], is responsible for the death of millions of pounds of food-sized channel catfish in the US [23]. Though both *E. ictaluri* and *A. hydrophila* pose serious threats to the US catfish industry [24,45,56] as well as global fish farming [46,62], highly efficient genome modification techniques have not been developed yet to study the virulence mechanisms and permit generation of avirulent vaccines for these two pathogens.

Though recombineering techniques are widely being used for genome modification of domesticated laboratory isolates such as *E. coli* strains, the implementation of these techniques for primary pathogenic isolates is quite challenging. In this study, we modified the available λ Red recombination tools [11,54] to generate markerless mutants of *E. ictaluri* and *A. hydrophila*. Several conjugally transferable and temperature-sensitive plasmids were constructed to facilitate the genome modification by recombineering and removal of antibiotic resistance marker followed by

Table 1
List of bacterial strains and plasmids used in this study.

Bacterial strains or plasmid	Features	References
E. coli		
SM10λpir	*thi-1thr leutonAlacYsupE recA*::RP4-2-TcT::Mu Km^r λ*pir*	[50]
BW25113/pKD46	F-, Δ*(araD-araB) 567*, Δ*lacZ4787*(::rrnB-3), λ$^-$, *rph-1*, Δ*(rhaD-rhaB) 568*, *hsdR514*, pKD46	[11]
BT340	F-, Δ*(argF-lac) 169*, ϕ80d*lacZ58*(M15), *glnV44*(AS), λ$^-$, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *spoT1*, *thiE1*, *hsdR17*, pCP20	[11]
BW25141/pKD4	F-, Δ*(araD-araB) 567*, Δ*lacZ4787*(::rrnB-3), Δ*(phoB-phoR) 580*, λ$^-$, *galU95*, Δ*uidA3::pir$^+$*, *recA1*, *endA9*(del-ins)::FRT, *rph-1*, Δ*(rhaD-rhaB) 568*, *hsdR514*, pKD4	[11]
"E. cloni" 10G	F$^-$*mcrA*Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA* ϕ80d*lacZ*ΔM15Δ*lacX74 araD139* Δ (ara,leu) 7697 *galU galK rpsL* (StrR) *nupG* λ$^-$ *tonA*	Lucigen Corp. WI
E. ictaluri		
Alg-08-183	Pathogenic isolates from diseased catfish	[22]
Alg-08-183 (pMJH46)	*E. ictaluri* strain Alg-08-183 with plasmid pMJH46	This study
R4383	Highly hemolytic *E. ictaluri* strain from diseased catfish	[59]
R4383 (pMJH46)	*E. ictaluri* strain R4383 with plasmid pMJH46	This study
Alg-08-183*ompLC::kanR*	Replacement of hemolysin *ompLC* gene with *kanR* gene	This study
Alg-08-183*ompLC::kanR* (pCP20)	*E. ictaluri* Alg-08-183*ompLC::kanR* with pCP20	This study
Alg-08-183 *drtA::kanR*	Replacement of hemolysin *drtA* gene with *kanR* gene	This study
Alg-08-183 *drtA::kanR* (pCP20)	*E. ictaluri* Alg-08-183 *drtA::kanR* with pCP20	This study
Alg-08-183Δ*ompLC*	In-frame deletion of *ompLC* gene	This study
Alg-08-183Δ*drtA*	In-frame deletion of *drtA* gene	This study
R4383*eihA::kanR*	Replacement of hemolysin *eihA* gene with *kanR* gene	This study
R4383*eihA::kanR* (pCP20)	*E. ictaluri* R4383*eihA::kanR* with pCP20	This study
R4383Δ*eihA*	In-frame deletion of hemolysin gene *eihA*	This study
A. hydrophila		
Ml09-119(pMJH46)	*A. hydrophila* ML09-119 with pMJH46	This study
Ml09-119(pMJH65)	*A. hydrophila* ML09-119 with pMJH65	This study
ML09-119*ymcC:cat* (pCMT-flp)	*A. hydrophila* ML09-119*ymcC:cat* with pCMT-flp	This study
ML09-119*ymcC:cat*	Replacement of *ymcA* gene with *cat* gene	This study
ML09-119Δ*ymcC*	Unmarked deletion of *ymcC* gene	This study
ML09-119*waaL::cat*	Replacement of *waaL* gene with *cat* gene	This study
ML09-119*iolA::cat*	Unmarked deletion of *iolA* gene	This study
ML09-119*hlyA::cat*	Replacement of *hlyA* gene with *cat* gene	This study
ML09-119Δ*hlyA*	Unmarked deletion of *hly* gene	This study
ML09-119*aerA::cat*	Replacement of *aerA* gene with *cat* gene	This study
ML09-119 *vgr3::cat*	Replacement of *vgr3* gene with *cat* gene	This study
ML09-119Δ*vgr3*	Unmarked deletion of *vgr3*gene	This study
ML09-119$_{3,822,477}$	Deletion of genetic region 3822,477..3,822,683 of ML09-119	This study
ML09-119 (pBBC2)	*A. hydrophila* ML09-119 with pBBC2	This study
Plasmids		
pACYC184	Cloning vector with p15A origin of replication	[63]
pKD46	Temperature-sensitive recombinogenic plasmid	[11]
pKD4	Template for recombineering substrate	[11]
pMJH46	Conjugally transferrable recombinogenic plasmid	This Study
pMJH65	Conjugally transferrable recombinogenic plasmid	This Study
pCMT-flp	Temperature-sensitive Flp recombinase plasmid	This Study
pMJH97	*cat-oriT-oriR* backbone plasmid for PCR-free cloning	This Study
pCP20	Temperature-sensitive Flp recombinase plasmid	[7]
pGNS-BAC	Conjugally transferable BAC vector	[27]

Table 2
List of primers used in this study.

Primer Name	Sequence in 5′ to 3′ direction
pKD4-ompLCf	AACTGGTAGATCATACCAACGCCAACGATGTTGTCGGTGCTGATACCGGCGTGTAGGCTGGAGCTGCTTC
pKD4-ompLCr	GTTCAAAAAATTCCCGATGGAATCAAATTAGGCAGTGGCAGGTGTCAAAACATATGAATATCCTCCTTAGT
ML44-RedF	ATGCTTACAACAAAAAATATGCCAGCCAATGCTGGGCTGGCAGCGTTTTCTGGTGTAGGCTGGAGCTGCTTC
ML44-RedR	TTAGCAAGGGGGAAGATGCTCTGGTGGTGGTCTGTTTTTCTGATGATAGCATATGAATATCCTCCTTAGT
ML-44R	TATGCAAGCTTATATAAGTGTAGTGCAGTATG
44expandedF	TATGCTCTAG AACTTAACTGTTGGTCATAG-3'
44expandedR	TATGCTCTAG AATATTCAACGGCATTAC-3'
Hemo-redF	TTCCTTTTAACTCTGCTTTGGCGCCCATGGGCGCTGATATGAGGCAATCTCTGTGTAGGCTGGAGCTGCTTC
Hemo-redR	ACGGCGGCCCGCAGGCCGCCGTTGAGGATGGATAACGTCGCCACTATCCGGTCATATGAATATCCTCCTTAGT
Takara-hemoF	TATGCAAGCTTCTCCTCATAGTGTGTCCGCAGT
Takara-hemoR	TATGCAAGCTTGCATTGACATAGGCGTTCATCT
H-RedtrackF	GATGTCTATCTGTTCAGCTC
H-RedtrackR	GTACGCAATACCAATAGTG
RE33-165F	TATGCAAGCTTGTAGTTCTTGCTGGTCTC
RE33-165R	TATGCAAGCTTGTAACGCAACATTCTAAC
k1	CAGTCATAGCCGAATAGCCT
k2	CGGTGCCCTGAATGAACTGC
kt	CGGCCACAGTCGATGAATCC
CatF	TATCGTGACTGACTGCTGCGTGTAGACTTCCGTTGAACT
CatR	ATGCAGATATCGCCTAATGAGTGAGCTAA
MobicatF	AGAGTGCTGACAGATGAG
MobicatR	ACGCAGCAGTCAGTCACGATAATGATGTGGTCTGTCCT
tetAR	CGACAGGAGCACGATCAT
tetAF	TGTAGCACCTGAAGTCAGC
Flp-pRhamF	CGC GAA CAG ATT GGA GGTCCACAATTTGGTATATTATGTA
Flp-pRhamR	GTG GCG GCC GCT CTA TTATATGCGTCTATTTATGTAGGA
UP-F-flp-oriR	ATGGCTTCCATGTCGGCAGAAT
DN-R-oriT	TTGGTGTATCCAACGGCGTCAGCCGGGCAGGATAGGTGAAGTAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGTCCCTTATTCGTTCCACTGAGCGTCAGACC-3'
Li-CCatF	T*G*G*G*GCAGTTGATGAAACATCGCGCAGCCTGCCGGCCCCACATGGCCTCGACAGCCGCTAGGTACC CGCTCCATGAGCTTATCGCGAAT
Li-AAAAR	A*T*G*C*ACTTTTTCATGCACAACCCCGGTGGGGCCGGGCTCTATCTGCCGTTCAACGCCTGGGGC CCTCCTGTTCAGCTACTGACG
CCatR-oriT	TTGGTGTATCCAACGGCGTCAGCCGGGCAGGATAGGTGAAGTAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGTCCCTTATTCGGCCGTCGACCAATTCTCATGTT
CatFseq	CTGGTTGCTACGCCTGAATAAGTG
p15AF	TCACATATTCTGCTGACGCACC
Li234R-HindII	AGT CTA AGC TTG CTC AAG CCA ACA ACC GCG AA
CCatR	GGC CGT CGA CCA ATT CTC ATG TT
ymcA-CM-1F	GCGACAAAAATAAGGCTGCCA
pMJH46SeqF	CGTCTACTCCGTTACAA
Mob-seqR	GGCTTCACCTTCAACC
pMJH46SeqR	AGTATGATCTCAATGGTTCG
Cat-SeqF	CAGAATGCTTAATGAATTAC
CatR-int	CATGCGATATCTAATGAATCGGCCAAC
FlpF1	CGCATTCACAGTTCTCCGCAAG
FlpR1	GTGCCTACTAACGCTTGTCT
FlpF2	CTTCGATCATTGGACCGCTG
FlpR2	CGAATCATCGGAAGAAGCAG
97seq1F	ACAAGACGTTGAGGCCACTATC
97seq2F	TTGGTCTGCGCGTAATCTCTTG
97seq3F	GGAACTGAGTGTCAGGCGTGGA
97seq4R	GGAGGCCAGATGTTGAGTCGCA
97Seq8R	GCAGCAGCCACTGGTAATTGA
97Seq7R	CAAGAGATTACGCGCAGACCA
97Seq5F	CAAGATGTGGCGTGTTACGGT
97Seq6F	GGACAGTGAAGAAGGAACACC
CCatF	CGCTCCATGAGCTTATCGCGAAT
AAAAF	CCTCCTGTTCAGCTACTGACG
BBBBR	TATCGATGATAAGCTGTCAA
Vgr3F	TCACCCGGCTTGCAGTGCCCCGCCTGATGGGGCTACACGACATCTCAGAGGCGCTCAGCGGTGTAGGCTGGAGCTGCTTC
Vgr3R	GATGGCACGAAAAAGGTCTGCGCGCAGGCCCTTGCTCCTTGAGCAGCGCCTCCATCGCCTTCATATGAATATCCTCCTTAGT
vgR3outR	GCATGCCGATGAACTCTTCAAGTG
vgR3outF	ATCCTGCGAAGTCTGACTTCACC
hlyA-RedF	T*A*A*T*ATGGTTATGCCGTGTTCGTTCATTGTTTAAATAGCTTGGCGTGATTCGACAAGGAGATAACAGTGTAGGCTGGAGCTGCTTC
hlyA-RedR	C*C*C*T*GCTCTGTCAGTGACTGGCCGGTGGCCCGAAGATGCGGGTGTAGGAGGTCAGGGTCCGTACGCCATATGAATATCCTCCTTAGT
hlyoutF	GCATGCCGAATCATCTCACCTTAGA
hlyoutR	CAGACCTTCTACAAGCTGGCGGAG
aero-RedF	T*G*C*C*GATATATAAGCGCTGGTGAATGTATGTCAATGTTCAATATATTGGGGTTGCTGTGTAGGCTGGAGCTGCTTC
aero-RedR	C*A*G*T*GCAAACAAAAACCGGGCCAGAGGCCCGGTTCCATCACTACAACGCACTGCCGATGGGAATTAGCCATGGTCC
Li234R	GCTCAAGCCAACAACCGCGAA
Li234*R	G*C*T*C*AAGCCAACAACCGCGAA
Ligase*F	G*A*C*C*AGCGCATTGAGAGAGAGG
Liup*F	A*C*T*T*AAGCTCGCCGAACTC
Lidn*R	T*G*A*T*TATGATGTAATGACTGG
Ligase-catF	T*G*G*G*GCAGTTGATGAAACATCGCGCAGCCTGCCGGCCCCACATGGCCTCGACAGCCGCTAGTAGACTTCCGTTGAACT
Ligase-catR	C*C*C*T*TTTATTATCTACCCAAGATATATGGTAATCTGCAGAAATTATGCTAGGAATGCATGGCCTAATGAGTGAGCTAA
Li-CatF	TGGGGCAGTTGATGAAACATCGCGCAGCCTGCCGGCCCCACATGGCCTCGACAGCCGCTAGTAGACTTCCGTTGAACT
Li-CatR	CCCTTTTATTATCTACCCAAGATATATGGTAATCTGCAGAAATTATGCTAGGAATGCATGGCCTAATGAGTGAGCTAA
O_2RecF	T*C*T*G*AGCGTAATCCATAGTCAAACCAGAAATTTTAAATTTAAGGATGTTGAATTTTGTAGACTTCCGTTGAACT
O_2RecR	T*A*G*A*GGAGGTATTACCCTCCTCTACTCCAAATTTTTATAAAAAATTCCGAATGTGAGCCTAATGAGTGAGCTAA

recombineering. In addition, we also developed a novel *in vivo* error-free cloning system that can be used to clone large fragments of genomic DNA without PCR amplification of the inserts and used to complement larger genomic regions.

2. Materials and methods

2.1. Bacterial strains and plasmids

The list of bacterial strains and plasmids used in this study is presented in Table 1. *E. ictaluri* and *A. hydrophila* strains were routinely grown on Trypticase Soy Broth (TSB) or Agar (TSA) medium at 28 °C and 30 °C, respectively. *E. coli* SM10λpir [50] was routinely used for the conjugal transfer of mobilizable plasmids to strains of *E. ictaluri* and *A. hydrophila* as previously described. *E. coli* BW25141 and BT340 [11] were received from the Yale University Genetic Stock Center. When antibiotic selection was required, bacterial growth media were supplemented with kanamycin (50 μg/ml), chloramphenicol (15 and 25 μg/ml for strains of *E. ictaluri* and *A. hydrophila*, respectively), tetracycline (10 μg/ml) and/or colistin (10 μg/ml).

2.2. Recombinant DNA techniques and conjugal transfer of recombinogenic plasmids

The list of primers used in this study is presented in Table 2. All oligonucleotides were purchased from Eurofins MWG Operon (Huntsville, AL). For cloning purposes, we routinely used electrocompetent *E. coli* ("E. cloni 10G", Lucigen Corp., Middleton, WI). PCR amplifications were carried out using EconoTaq DNA polymerase (Lucigen Corp.), *Pfu* DNA polymerase (Life Technologies, Grand Island, NY) and TaKaRa Ex Taq (Clontech, Mountain View, CA) as appropriate. Genomic DNAs and plasmids were extracted using the E.Z.N.A. DNA Isolation Kit (Omega Biotek, Atlanta, GA) and FastPlasmid Mini Kit (5 Prime, Gaithersburg, MD), respectively. Restriction enzymes and T4 DNA Ligase (Quick ligase) used for restriction digestion of DNAs and cloning, respectively, were purchased from New England Biolabs (Ipswich, MA). Restriction digested DNAs with sticky ends were blunt-ended using a DNA Terminator kit (Lucigen Corp.). Digested DNAs and ligation mix were purified using DNA Clean and Concentrator-5 (Zymo Research, Irvine, CA). DNA concentrations were quantified using a Qubit 2.0 Fluorometer (Life Technologies). The mobilizable recombinogenic plasmids pMJH46 and pMJH65, and *flp* recombinase plasmid pCMT-flp were introduced into *E. coli* SM10λpir by electroporation according to a previously published method [47]. Plasmids were conjugally transferred into *E. ictaluri* and *A. hydrophila* by filter mating experiments according to the methods described previously [35]. *E. ictaluri* and *A. hydrophila* transconjugants were selected on LB plates supplemented with chloramphenicol and colistin, or tetracycline and colistin, respectively. The introduction of plasmids into *E. ictaluri* or *A. hydrophila* was confirmed by their growth in the presence of appropriate antibiotics and by conducting PCR with a plasmid-specific primer set.

2.3. Construction of broad host range recombinogenic plasmids

A list of plasmids used in this study is presented in Table 1. The mobilizable plasmid pMJH46 was constructed by introducing the *oriT* sequence and chloramphenicol acetyltransferase (*cat*) gene into the recombinogenic plasmid pKD46 [19] which contains an arabinose-inducible λ-Red cassette (*exo*, *bet* and *gam* genes) required for recombineering (Fig. 1). The *oriT* sequence and *cat* gene were PCR amplified from pGNS-BAC [27] using primers MobicatF and MobicatR, and CatF and CatR, respectively. Amplicons for the *oriT* sequence and *cat* gene were fused by splicing by

overlap extension (SOE) PCR [52] using primers MobicatF (forward) and CatR (reverse). The *oriT-cat* cassette and pKD46 plasmid were digested with EcoRV and NcoI, respectively. NcoI digested pKD46 plasmid was blunt-ended and ligated to *oriT-cat* cassette using a DNA Terminator kit (Lucigen Corp., Middleton, WI) and T4 DNA ligase (Promega, WI), respectively. The ligation mixture was then transformed into electrocompetent *E. coli* (E. cloni 10G, Lucigen Corp.) for cloning. Transformants were selected on 2 × YT medium supplemented with ampicillin and chloramphenicol after incubation overnight at 30 °C. The introduction of the *oriT-cat* cassette into pKD46, resulting in pMJH46, was confirmed by PCR and sequencing as described below. To construct the recombinogenic plasmid pMJH65, plasmid pMJH46 was digested with BstZ17I and SfiI, and blunt-ended using the DNA Terminator kit. A tetracycline resistance gene (*tetA*) cassette was PCR amplified from pACYC184 using primers tetAF and tetAR and ligated to blunt-ended pMJH46 using T4 DNA ligase. The ligation mixture was then transformed into electrocompetent *E. coli* (E. cloni 10G, Lucigen Corp.) for cloning. Transformants were selected on 2×YT medium supplemented with tetracycline after overnight incubation at 30 °C. The construction of plasmid pMJH65 was confirmed by PCR and sequencing as described below.

2.4. Construction of conjugally transferable Flp plasmid pCMT-flp

The *flp* gene, which is required for FRT mediated site-specific recombination [7], was PCR amplified from pCP20 using primers Flp-pRhamF and Flp-pRhamR and was cloned into pRham N-His SUMO vector (Lucigen Corp.) under the control of the *rhaPBAD* promoter. The resulting plasmid pRham-flp was then digested with XbaI and blunt-ended in order to insert a tetracycline resistance gene (*tetA*) which was PCR amplified from pMJH65 using primers tetAF and tetAR. After cloning this *tetA* cassette into the pRham-*flp* plasmid, resulting in plasmid pRham-*flp-tetA*, the *flp-tetA* cassette was digested with AlwNI and BsaAI, and blunt-ended for cloning into *repA101*-oriR101 cassette which was PCR amplified from pMJH65 using primers UP-F-flp-oriR and DN-R-oriT. After cloning *flp-tetA* into *repA101*-oriR101 cassette, the construction of the resulting plasmid pCMT-flp was confirmed by sequencing as described below. To determine the efficacy of pCMT-flp plasmid in excision of an antibiotic resistance cassette flanked by FRT sequences, pCMT-flp was transferred into strains of *A. hydrophila* mutants by conjugation as described above.

2.5. Preparation of linear double stranded DNA (dsDNA) substrate for recombineering

The linear dsDNA fragments used for deletion of the *ompLC* gene from *E. ictaluri* with recombineering were generated by PCR amplification of the kanamycin resistance gene (*kanR*) cassette with its flanking FRT sequences using plasmid pKD4 as a template [11]. All other linear dsDNA used for deletion of *E. ictaluri* genes *eihA* and *dtrA* were PCR amplified from a *kanR* cassette located within the genome of *E. ictaluri* Alg-08-183*ompLC::kanR* mutant generated in this study by recombineering. Likewise, the linear dsDNA substrate used for recombineering in *A. hydrophila* was generated by PCR amplification of the *cat* gene with its flanking FRT sequences integrated within the genome of *A. hydrophila* ML09-119 (see below). Recombineering primers contained 50–60 bp of homology to the targeted genes at their 5′ ends and 20–22 bp of homology to the *cat* cassette at their 3′ ends. Primers were modified with four consecutive 5′ phosphorothioates bonds when appropriate to reduce the chance of degradation by exonucleases during recombination. To introduce ∼250 and ∼500 bp homologous arms on either ends of the recombineering substrates for the determination of the effect of length homology in recombination
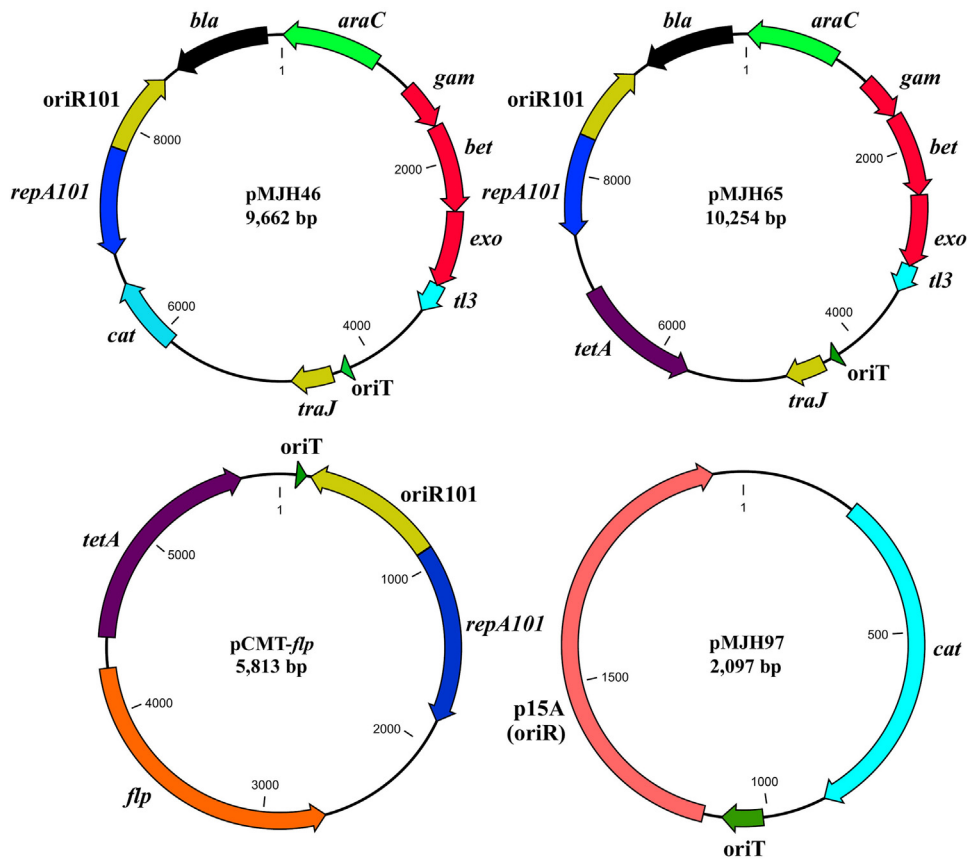
Fig. 1. Schematic maps of conjugally transferable recombinogenic and *flp* recombinase plasmids constructed in this study. The *oriT* sequence cloned into these plasmids facilitates the conjugal transfer of these plasmids using appropriate donor *E. coli* strain. Red recombinogenic plasmids pMJH46, pMJH65 and flp recombinase plasmid pCMT-flp are easily cured after heat induction at 37 °C due to temperature sensitive *repA101* gene. Plasmid maps were generated by CLC Genomics Workbench (version 4.9).

frequency, primers were designed to anneal ~250 and ~500 bp upstream and downstream, respectively, of the *cat* gene of *A. hydrophila* ML09-119 *waaL::cat* mutant generated by recombineering in this study. PCR amplification of the respective antibiotic resistance gene cassette using these gene-targeted primers was performed using high fidelity Takara Ex Taq Polymerase (Clontech) and EconoTaq PLUS GREEN (Lucigen Corp.). At least 10 positive PCR amplicons of 50 μl volume were pooled together and purified by phenol–chloroform extraction followed by ethanol precipitation [47] or using the Wizard DNA Clean-Up System (Promega, Madison, WI). Purified PCR products were resuspended in nuclease-free water and used for transformation into electrocompetent *E. ictaluri* and *A. hydrophila* strains harboring recombinogenic plasmids pMJH46 and pMJH65, respectively.

2.6. Deletion of E. ictaluri and A. hydrophila genes by recombineering

Electrocompetent *E. ictaluri* and *A. hydrophila* harboring recombinogenic plasmids pMJH46 and pMJH65, respectively, were prepared as described follows. *E. ictaluri* strains were grown in TSB at 28 °C for overnight in the presence of chloramphenicol, whereas *A. hydrophila* was grown at 30 °C for overnight in TSB supplemented with tetracycline. Cultures were then diluted 1:70 in 40 ml of Super Optimal broth (SOB) medium supplemented with appropriate antibiotics and 10 mM L-arabinose, and grown with vigorous shaking until the OD_{600} reached to 0.45 or 0.6 for *E. ictaluri* and *A. hydrophila*, respectively. Cells were harvested by centrifugation at $5000 \times g$ for 8 min at 4 °C, washed three times with ice-cold 10% glycerol and finally cells were concentrated 400-fold by resuspending with 100 μl of ice-cold GYT (10% glycerol, 0.125% yeast

extract and 0.25% tryptone) medium or 10% glycerol. Freshly prepared electrocompetent cells were immediately used for electroporation. For deletion of targeted genes from *E. ictaluri* using recombineering, a dsDNA substrate of 10 μg were mixed with 50–55 μl of electrocompetent cells in a pre-chilled electroporation cuvette (0.1-cm gap), and pulsed at 1.8 kV with 25 μF and 200 W using an Eppendorf Electroporator 2510 (Hamburg, Germany). For *A. hydrophila*, the same electroporation procedures were followed with the exception that cells were pulsed at 1.2 kV. Immediately after electroporation, 950 μl of SOC supplemented with 10 mM L-arabinose was added and incubated at an appropriate temperature with vigorous shaking for at least 4 hrs for *E. ictaluri* and overnight for *A. hydrophila*. Cells were then spread onto 2 × YT agar plates supplemented with kanamycin and chloramphenicol for *E. ictaluri* and *A. hydrophila*, respectively, and incubated at an appropriate temperature to obtain mutants with the targeted deletions. Mutants grown on antibiotic selective plates were purified by streaking on TSA plates for isolated colonies. The correct deletions of the targeted genes were confirmed by PCR and/or sequencing as previously described [11]. To determine the effect of (1) phosphorothioate-modified primers, (2) the size of the gene-specific region of homology and (3) the concentration of the dsDNA substrates on recombination frequencies, each experiment was repeated independently at least three times.

2.7. Flp-mediated excision of antibiotic resistance gene cassettes to generate unmarked mutants

Before removal of the antibiotic resistance gene cassettes using Flp/FRT mediated recombination, recombinogenic plasmids were

cured from the mutants of *E. ictaluri* and *A. hydrophila*. Plasmid pMJH46 was cured from *E. ictaluri* mutants by growing cells on TSB medium at 28 °C until the OD_{600} reached to 1.0 and then cells were subjected to heat induction at 43 °C for 1 h with shaking at 250 rpm. Heat-induced cultures were serially diluted in sterile water and spread for isolated colonies onto BHI Blood Agar plates that were then incubated at 28 °C for 36 h. To cure plasmid pMJH65 from *A. hydrophila* mutants, cultures were grown in TSB broth at 37 °C overnight and streaked onto TSA plates for isolated colonies. The loss of plasmid pMJH46 and pMJH65 from *E. ictaluri* and *A. hydrophila* mutants were confirmed by determining the lack of ability of individual mutant colonies to grow on TSA plates supplemented with chloramphenicol and tetracycline, respectively. Plasmid pCP20 that contains the Flp recombinase [7] required for FRT sequence-specific recombination was electroporated into *E. ictaluri* mutants according to the methods described above. *E. ictaluri* mutants harboring pCP20 were selected on 2 × YT agar plates supplemented with chloramphenicol. These *E. ictaluri* mutants were grown in TSB at 28 °C until OD_{600} of 1.0 and temperature was shifted by incubating at 37 °C for 1 h with shaking at 250 rpm to induce the removal of kanamycin resistance gene cassette by FLP recombinase. To obtain isolated colonies diluted cultures were plated onto BHI Blood Agar plates and incubated at 28 °C for up to 36 h. Flp recombinase plasmid pCMT-*flp* constructed in this study was conjugally transferred to *A. hydrophila* mutants as described above and induced for the removal of chloramphenicol resistance gene cassette by incubating at 37 °C. Induced cultures were streaked onto TSA plates and colonies grown on non-selective plates that subsequently failed to grow on antibiotic selective plates were tested by PCR and sequencing to confirm the Flp-mediated excision of antibiotic resistance gene cassette which was introduced by recombineering.

2.8. Cloning large genomic inserts without PCR amplification of the targeted genetic locus

To construct a small, conjugally transferrable, and low copy-number plasmid backbone, the *cat* gene and p15A origin of replication (*oriR*) were PCR amplified using primers Li-CCatF and CCatR-oriT and CatFseq and Li-AAAAR, respectively, from the genome of *A. hydrophila* ML09-119*hlyA::cat* (generated in this study) and plasmid p1R17 (unpublished) with p15A of pACYC184 origin, respectively. The reverse primer CCatR-oriT used for amplification of the *cat* gene contains 87 bp of *oriT* sequence (Table 2) to facilitate the conjugal transfer of large insert clones to Gram-negative bacteria. The amplicons of *cat-oriT* cassette and p15A (*oriR*) were fused together to construct a 2097 bp plasmid backbone *cat-oriT-oriR* (pMJH97) using SOE PCR with outermost primers Li-CCatF and Li-AAAAR. To clone the *ymcABC* genetic cluster (unpublished data, manuscript in preparation) of *A. hydrophila* ML09-119, the pMJH97 plasmid backbone was PCR amplified using primers Li-CCatF and Li-AAAR that are homologous to the nucleotide regions 3,497,544-3497603 and 3,499,203-3499265, respectively, of the *A. hydrophila* ML09-119 genome [53]. These regions correspond to a specific region which is upstream of the *ymcABC* genetic cluster in the *A. hydrophila* ML09-119 genome. Purified PCR products were electroporated into *A. hydrophila* ML09-119 harboring plasmid pMJH65 for genomic integration into the targeted regions by recombineering. Colonies selected on 2 × YT plates containing chloramphenicol were subjected to PCR to confirm the correct integration of the pMJH97 backbone plasmid into the genome using primers p15AF and Li234R-HindII, and amplicons of the expected size were selected for sequencing. Once the correct integration of pMJH97 into the genome of *A. hydrophila* ML09-119 was confirmed by PCR and sequencing, genomic DNA was extracted from ML09-

119::*cat-oriT-oriR* and restriction digested with BbvCI and NotI. Blunt-ended and purified genomic DNA fragments were self-ligated using T4 DNA ligase and electroporated into *E. coli* (E. cloni 10G, Lucigen Corp.) for cloning. Clones were selected on 2 × YT plates with chloramphenicol and the cloned plasmid pBBC2 was verified by PCR and sequencing using primers CCatR and ymcA-CM-1F for the presence of the complete *ymcABC* genetic cluster as an insert. Once the complete *ymcABC* cloning was confirmed, the pBBC2 was introduced into *E. coli* SM10λpir by electroporation. The plasmid was conjugally transferred into *A. hydrophila* ML09-119 as described above. Ten transconjugants which were grown on 2 × YT plates supplemented with chloramphenicol and colistin were double purified and subjected to PCR to confirm pBBC2 mobilization into *A. hydrophila* ML09-119 using primers CCatR and ymcA-CM-1F.

2.9. Sequencing of conjugally transferable recombinogenic and flp plasmids

The constructions of plasmids pMJH46 and pMJH65 were confirmed by PCR and sequencing using primers pMJH46SeqF, Mob-seqR, pMJH46SeqR and Cat-SeqF for plasmid pMJH46 and primers CatF and CatR-int for plasmid pMJH65 (Table 1). Plasmid pCMT-flp was sequenced using Illumina MiSeq according to methods described previously [43]. The gaps between the contigs obtained after assembling of Illumina MiSeq sequence reads of pCMT-flp were filled by PCR and sequencing using primers FlpFa, FlpR1, FlpF2 and FlpR2 (Table 2). The sequencing of the linear cassette pMJH97 (*catR-oriT-oriR*) integrated into the *A. hydrophila* genome was confirmed by PCR and sequencing using primers 97Seq1F, 97Seq2F, 97Seq3F, 97Seq4R, 97Seq8R, 97Seq7R, 97Seq5F, 97Seq6F, p15AF, CCatF, AAAAF and BBBBR (listed in Table 2).

2.10. Nucleotide sequence accession and Addgene deposition ID numbers

The sequences of plasmids pMJH46, pMJH65, pMJH97 and pCMT-flp were deposited to the NCBI GenBank sequence database under accession numbers JQ070344, KF195927, KT072897, and KT072898, respectively. Plasmids pMJH46, pMJH65 and pCMT-flp were deposited with Addgene (https://www.addgene.org/) with the plasmid numbers 67,272, 67,273 and 67,274, respectively.

3. Results

3.1. Construction of conjugally transferable recombinogenic plasmids

The expression of *exo*, *bet* and *gam* within bacterial cells substantially improves their recombination frequencies that can be exploited to modify bacterial genomes by recombineering [11]. Though published reports indicate that some *E. ictaluri* strains are capable of accepting foreign DNA of up to 45 kb by electroporation [23], our repeated attempts failed to introduce the recombinogenic plasmid pKD46 [11] into primary disease isolates of *E. ictaluri* or *A. hydrophila*. To introduce the recombinogenic λ-Red cassette into *E. ictaluri*, a mobilizable plasmid was constructed by introducing the 'mob cassette' (*oriT* region, *traJ* and *traK*) along with a chloramphenicol resistance (*cat*) gene into pKD46, resulting in plasmid pMJH46 (Fig. 1, accession no. JQ070344). The *cat* gene introduction broadens the applicability of this plasmid since some *E. ictaluri* strains are intrinsically resistant to ampicillin [58]; therefore, the original plasmid pKD46 expressing the *bla* gene is incompatible for these *E. ictaluri* isolates. In this study, we successfully transferred recombinogenic plasmid pMJH46 into different *E. ictaluri* strains by conjugation with *E. coli* SM10λpir. In subsequent studies, the pMJH46 plasmid was modified by replacing the *cat* gene with *tetA*

to construct recombinogenic plasmid pMJH65 (Fig. 1, accession no. KF195927) which allows the use of the *cat* gene as a recombineering substrate. The plasmid pMJH65 was successfully introduced into highly virulent catfish isolate *A. hydrophila* ML09-119 [53] in order to generate genomic modifications through recombineering.

3.2. Deletion of E. ictaluri and A. hydrophila genes by recombineering

To determine the feasibility of using this recombineering system in *E. ictaluri*, we deleted the *ompLC* gene that is required for phage ΦeiAU-183 attachment to *E. ictaluri* strain Alg-08-183 [22]. The PCR screening of colonies grown on antibiotic selection plates showed that 1% colonies were true mutants (data not shown). Unfortunately, a large number of colonies grown on 2 × YT plates supplemented with kanamycin were determined to be false positive even though the suicide plasmid pKD4 [10] used as template was treated with *DpnI* before electroporation into *E. ictaluri*. To avoid the occurrence of background colonies, we subsequently used the genomic DNA of the *E. ictaluri* Alg-08-183 *ompLC::kanR* mutant as a PCR template for amplification of the kanamycin resistance gene cassette. Using this chromosomal template to prepare amplicons, we obtained at least ten colonies per experiment, of which ~80% of them were true mutants (Fig. 2). In addition to *ompLC* of *E. ictaluri* Alg-08-183, we deleted two additional genes that included *dtrA* of *E. ictaluri* Alg-08-183, and *eihA* of *E. ictaluri* R4383 [59] (Fig. 2). In this study, using a recombineering approach, we also deleted seven different genes from the primary disease isolate *A. hydrophila* ML09-119 (Table 1). PCR and sequencing confirmed that all genes that were targeted for deletion from *E. ictaluri* and *A. hydrophila* strains were successfully deleted by recombineering. As a control experiment, *A. hydrophila* ML09-119 (pMJH65) and this same strain without the presence of the recombineering plasmid were both subjected to electroporation with equal amounts (900 ng) of a *waaL::cat* PCR construct (Table 1), and only in the presence of pMJH65 were any transformants obtained at a frequency of 0.45 ± 0.27 transformants per ng of amplicon DNA.

3.3. Effects of primer modification, length of homology and dsDNA substrate concentration on recombination frequency

To determine the effect of strand protection through primer modifications on recombination frequencies in *A. hydrophila* ML09-119, four different primers combinations were used for the preparation of dsDNA substrates to delete the *waaL* gene of *A. hydrophila* ML09-119 [53]. In the type "+/+"primer combination, both the forward and reverse primers (Ligase-catF and Ligase-catR, in Table 2) were modified with four consecutive 5′-phosphorothioate bonds, whereas in the type "−/−" primer combination both the forward and reverse primers (Li-catF and Li-catR) were unmodified. In the type "+/−" primer combination, only the forward primer (Ligase-catF) was modified, whereas in the type "−/+" primer combination only the reverse primer (Ligase-catR) was modified with four consecutive 5′-phosphorothioate bonds. In the latter two cases, the alternative primers were unmodified. We found that dsDNA substrate prepared with both the leading and lagging strand-specific phosphorothioate modified primers (type "+/+"in Fig. 3B) provided significantly more mutants, whereas three other combinations did not affect recombination frequency (Fig. 3B). Once we determined that modified primers provided significantly more mutants, all of our subsequent recombineering experiments in *A. hydrophila* were carried out using both modified primers.

To determine the effect of the length of the gene-specific regions of homology of the dsDNA substrate on recombination efficiency, three different dsDNA substrates that included approximately 60 bp, 250 bp and 500 bp of homologous sequence at both the 5′ and 3′ ends were used for targeted deletion of the *waaL* gene
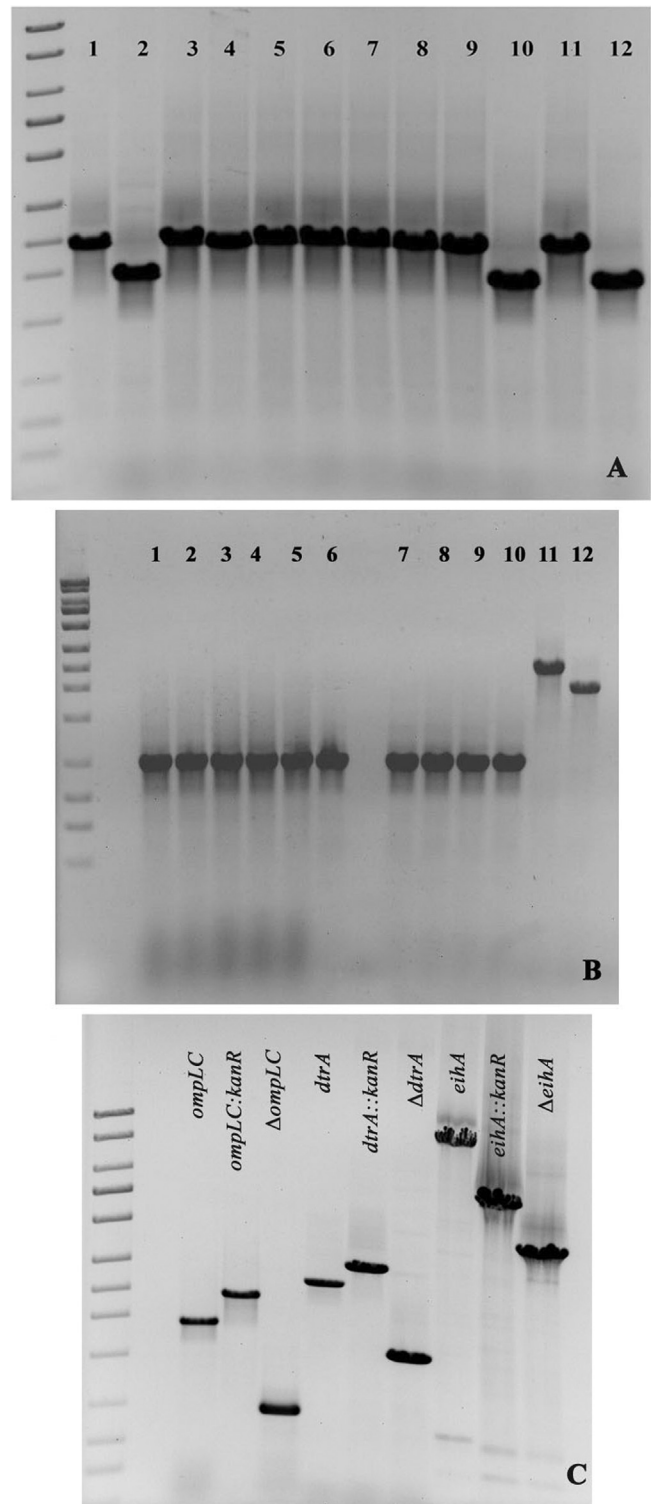
Fig. 2. Targeted deletion of *E. ictaluri* genes *ompLC*,*dtrA* and *eihA* by recombineering. (Panel A) Colonies gown on 2 × YT plates supplemented with kanamycin were selected for PCR screening of *ompLC* gene deleted mutants. Lanes 1, 3–9 and 11 represent the PCR products of *ompLC* gene mutants disrupted with the *kanR* gene (*ompLC::kanR*) and lanes 2, 10 and 12 represents the PCR product of wild type *ompLC* gene of *E. ictaluri* strain Alg-08-183. (Panel B) Removal of the kanamycin resistance marker using the Flp recombinase of plasmid pCP20. PCR screening of *E. ictaluri* mutants plated after temperature induction showed that all tested mutants had lost the antibiotic resistance marker. (Panel C) PCR confirmation of deletion of the *ompLC* and *drtA* genes from *E. ictaluri* strain Alg-08-183 and *eihA* from *E. ictaluri* strain R4383.
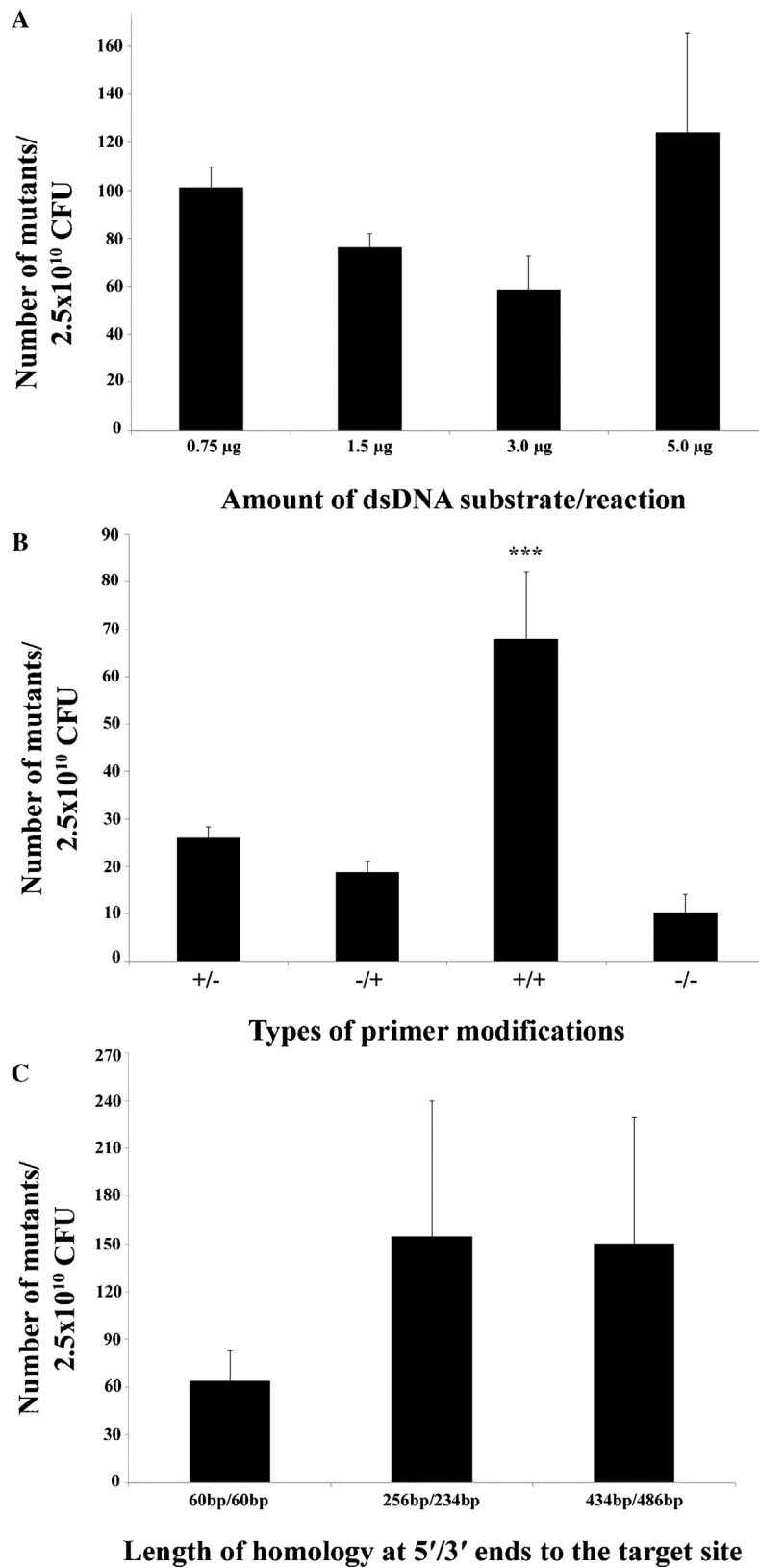
Fig. 3. Determination of recombination frequency in *A. hydrophila*. (Panel A) The effect of dsDNA substrate concentration on recombination frequency in *A. hydrophila* was determined using four different dsDNA substrate concentration ranging from 0.75 μg to 5.0 μg per recombineering experiment. (Panel B) Four different primer combinations were generated using modified and unmodified primers. Modified primers included four consecutive phosphorothioate bonds at the 5′ end of the primers. Type "−/−" used unmodified primers as a negative control, type "+/−" included modification of the forward primer but not the reverse primers, type "−/+" included modification to the reverse but not forward primer, and type "+/+" included phosphorothioate bonds in both primers. The latter condition in which both primers were modified provided significantly more mutants than any other types of dsDNA substrates used for recombineering (***p-value = 0.0026). (Panel C) The effect of varying the length of the homologous regions of the dsDNA substrate to the targeted chromosomal site on the recombination frequency was determined using approximately 60 bp, 250 bp and 500 bp of homologous sequence at both the 5′ and 3′ ends. The average number of mutants obtained was derived from three independent recombineering experiments.

of *A. hydrophila* ML09-119 [53]. The number of mutants obtained from this experiment demonstrated that the recombination frequencies were not significantly different in *A. hydrophila* ML09-119 due to the varying length of homologous arms flanking to the targeted gene (Fig. 3C).

To determine the effect of dsDNA substrate concentration on recombination frequencies in *A. hydrophila*, we used four different concentrations of dsDNA substrate that included 0.75, 1.5, 3.0 and 5.0 µg of dsDNA substrate for each recombineering experiment. Our findings demonstrated that increasing the dsDNA substrate concentrations did not change the recombination frequency significantly in *A. hydrophila* ML09-119 (Fig. 3A). The number of mutants we routinely obtained in this experiment was within the range of approximately 30–200 per recombineering reaction.

3.4. Removal of antibiotic resistance cassette by Flp recombinase

The temperature induction of *E. ictaluri* Alg-08-183*ompLC::kanR, dtrA::kanR* and *E. ictaluri* R4383 *eihA::kanR* mutant at 43 °C for 1 h followed by plating on BHI blood agar plates resulted in the curing of the recombinogenic plasmid pMJH46 (data not shown). We found that only highly rich medium such as BHI supplemented with 5% Sheep Blood, unlike TSA, supported the growth of the high temperature-induced *E. ictaluri* strains. The introduction of plasmid pCP20 containing the Flp recombinase by electroporation [7] followed by their growth at 37 °C resulted in removal of the antibiotic marker from the *E. ictaluri ompLC* mutant (Fig. 2B). PCR amplification of the targeted genes with their flanking primers indicated a 100% frequency for removal of the antibiotic selection marker. The antibiotic resistance markers from the *E. ictaluri dtrA* and *eihA* mutants were also removed using the Flp recombinase (Fig. 2C). We found that, in addition to the removal of the antibiotic resistance marker, heat induction efficiently cured the plasmid pCP20 from all mutant colonies tested. Cured mutants lacking the antibiotic resistance cassette could be subsequently targeted for deletion of additional genes. Since genes from *A. hydrophila* were replaced using the *cat* gene cassette, plasmid pCP20 containing the *cat* gene was not compatible for conducting Flp/FRT mediated recombination in *A. hydrophila* mutants. Therefore, we constructed a new *flp* recombinase plasmid pCMT-*flp* (Fig. 1D) with a tetracycline selectable marker. This plasmid was conjugally transferred into *A. hydrophila* mutants for markerless mutant construction. The screening of *A. hydrophila* mutants harboring the pCMT-*flp* plasmids for lack of growth in the presence of chloramphenicol resulted in more than 10% of the mutants with documented loss of the antibiotic resistance cassette (data not shown).

3.5. Cloning without PCR amplification of large inserts

Since the cloning of large inserts using traditional cloning techniques is challenging and PCR amplification of the targeted inserts can introduce unwanted mutations, we developed a novel technique to clone large genomic inserts of *A. hydrophila* that does not require PCR amplification of the targeted insert (Fig. 4). As a proof of concept of this technique, we targeted for cloning the 3.6 kb *ymcABC* operon of *A. hydrophila* strain ML09-119 for cloning. For this purpose, we constructed a small conjugally transferrable low copy-number plasmid backbone (pMJH97) which was integrated contiguous to the *ymcABC* operon of *A. hydrophila* ML09-119 by recombineering (data not shown). We confirmed the correct integration of the plasmid backbone (pMJH97) upstream of the *ymcABC* operon by PCR and sequencing. Restriction digestion of the genomic DNA isolated from the integrant and self-ligation followed by electroporation resulted in hundreds of chloramphenicol-resistant *E. coli* clones on selective plates. Two of the clones

selected for PCR and sequencing confirmation demonstrated that the intact *ymcABC* operon was cloned into the plasmid pMJH97 (data not shown). This plasmid was conjugally transferred into *A. hydrophila* ML09-119 to determine its conjugal transferability; screening of ten transconjugants using PCR demonstrated that all of the transconjugants harbored plasmid with an intact *ymcABC* operon insert (data not shown).

4. Discussion

The genetic manipulation of primary pathogenic isolates, compare to domesticated laboratory isolates, can be challenging due to many factors including antibiotic resistance [16,30], poor recombination efficiency and wide-spread occurrence of restriction-modification systems [37,54]. Our attempts to genetically modify the fish pathogens *E. ictaluri* and *A. hydrophila* were inhibited due to our inability to introduce the λ Red recombineering system into these bacterial isolates. Similar difficulties were observed by several other researchers who reported reduced transformation efficiency of pKD46 in *E. coli* by electroporation [48], demonstrating the need for an alternative route to introduce the recombineering system, i.e., via conjugation. In this study we describe the development of a fast, efficient, and reliable technique for genetic modification of *E. ictaluri* and *A. hydrophila* (and presumably other Gram-negative bacteria) using a recombineering system that is readily transferrable by conjugation. The introduction of a mob cassette to pKD46 [11] permitted the resulting plasmid pMJH46 to transfer into different *E. ictaluri* strains by conjugation. Additional modified recombinogenic plasmids were constructed to make it compatible for gene deletion in a highly virulent strain of *A. hydrophila*. Furthermore, we demonstrated the applicability of this method by creating multiple mutants in *E. ictaluri* and *A. hydrophila*.

Our first experiments using recombineering in *E. ictaluri* unfortunately were plagued by a large number of background colonies on the antibiotic selection plates that were not successful recombinants. These results were obtained even though we used suicide plasmid pKD4 as a template for PCR amplification of the antibiotic cassette and treated the DNA with *DpnI* treatment, as had been shown to reduce the number of background colonies [49]. An alternative solution to reducing the high background of antibiotic resistant colonies was to use genomic DNA isolated from a successful genomic integrant (*E. ictaluri* Alg-08-183*ompLC::kanR*) constructed in this study as a template for PCR of the recombineering construct. Therefore, all of our subsequent recombineering experiments for gene deletion in *E. ictaluri* and *A. hydrophila* used genomic DNA as template for PCR amplification of the respective antibiotic resistance gene cassettes.

We were able to use the Flp recombinase encoded on the temperature-sensitive plasmid pCP20 [7] to successfully remove a FRT-flanked antibiotic resistance cassette used for genome modification in *E. ictaluri*. Before introducing pCP20 into *E. ictaluri* mutants, pMJH46 was cured by heat induction since both plasmids contain the *cat* gene. Unlike *E. coli* [11], *E. ictaluri* mutants required a highly rich medium (BHI supplemented with 5% sheep blood) to recover after heat-induction at 43 °C, which may be due to the mesophilic growth temperature (28 °C) of *E. ictaluri*. Because of antibiotic resistance marker incompatibility, a new conjugally transferable *flp* recombinase plasmid, pCMT-*flp*, was constructed that can efficiently remove FRT-flanked antibiotic resistance gene cassettes from mutants of *A. hydrophila*.

In addition to developing techniques for genetic modification in *E. ictaluri* and *A. hydrophila*, we devised a technique for cloning large fragments of bacterial genomes without PCR amplification of the targeted region. Similar in concept to the VEX-capture system that uses a lox/Cre site-specific recombination system [60], or the use of an *in vivo* recombineering method [55], these cloning
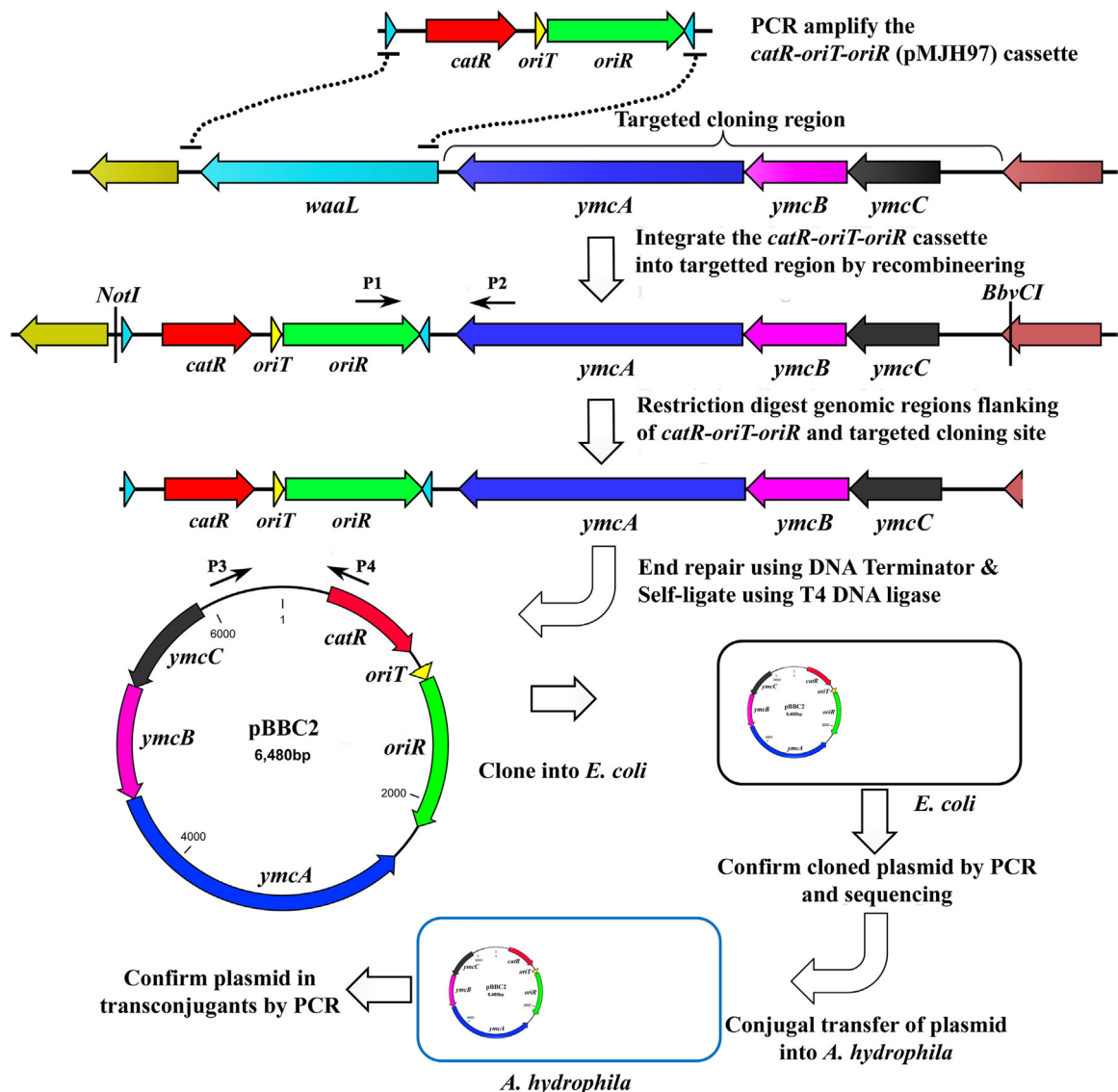
Fig. 4. Strategy for PCR-free cloning of large bacterial genetic regions. The major steps of cloning large genetic inserts are indicated. The *catR-oriT-oriR* (pMJH97) cassette was PCR amplified using primer pairs with 50–60 bp homologous sequence at their 5′-ends specific to the targetred site. Depending on the choice of restriction enzymes, the resulting dsDNA substrate can be integrated upstream or downstream of the targeted site of the genome using the recombineering system. Once the *catR-oriT-oriR* (pMJH97) cassette integration into the genome was confirmed by PCR and sequencing using primers P1 and P2, the genomic DNA of integrants was restriction digested with an appropriate restriction enzyme to clone into *E. coli* after self-ligation using T4 DNA ligase. The cloning of the correct insert into the plasmid pMJH97 was verified by PCR and sequencing using vector and insert specific primers P3 and P4, respectively. The plasmids with cloned inserts were then readily transfered to other Gram-negative bacterial strain by *oriT* sequence-mediated conjugal transfer using an appropriate donor strain.

systems are advantageous in allowing the cloning of larger fragments of genomic DNA without the need for PCR amplification, given the difficulties in producing larger amplicons and the potential for incorporating PCR-mediated errors. This method was validated by the cloning of the *A. hydrophila* genetic operon *ymcABC*, as an example of this method that can overcome the shortcomings of PCR-based methods for the cloning and conjugal transfer of genetic elements. The maximum possible size of the cloned region will depend on multiple factors, such as the presence of suitable restriction sites and the efficiency of conjugal transfer, but would be expected to be theoretically suitable for genomic regions such as genomic islands, prophages, and other genetic clusters.

We have described a highly efficient and rapid procedure for the generation of markerless mutants in *E. ictaluri* and *A. hydrophila* by recombineering. The newly constructed conjugally transferable recombinogenic plasmids pMJH46 and pMJH65 and recombinase plasmid pCMT-*flp* can presumably be used for other Gram-negative bacteria for generating markerless mutants, especially for bacterial isolates that are recalcitrant to electroporation. Finally, the development of a PCR-free system for cloning and transfer will facilitate cloning and complementation of much larger genetic elements.

Acknowledgements

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