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Genome modifications and cloning using a conjugally transferable recombineering system

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ABSTRACT

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*, *ΔlacZ4787*(::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*, *ΔlacZ4787*(::rrnB-3), *Δ(phoB-phoR) 580*, λ$^-$, *galU95*, *ΔuidA3::pir$^+$*, *recA1*, *endA9*(del-ins)::*FRT*, *rph-1*, *Δ(rhaD-rhaB) 568*, *hsdR514*, pKD4	[11]
"E. cloni" 10G	F$^-$*mcrAΔ(mrr-hsdRMS-mcrBC) endA1 recA* ϕ80d*lacZ*ΔM15Δ*lac*X74 *araD*139 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 *dtrA* 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 *dtrA* 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
ML09-119*ΔymcC*	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]