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Recombineering and Conjugation as Tools for Targeted Genomic Cloning

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1. Introduction

The ability to obtain DNA clones of genes that normally reside in microbial genomes was a huge technical advance in molecular biology. At first, cloning genes utilized approaches involving the complementation of mutants or the screening of genomic libraries to find sequences that hybridized to homologous DNA probes. Typically, this involved using restriction enzymes to clone random genomic fragments followed by subcloning of a smaller piece of the original clone. Then the development of PCR and genomic sequencing allowed specific genomic sequences to be amplified and cloned with more convenience. Now genes are able to be synthesized “from scratch” and ordered from various companies or institutions. However, if many genes contained on a contiguous large genomic segment are required to be cloned, significant technical barriers exist. For the purposes of this discussion, we will establish that a “large” genomic segment constitutes greater than 10 kilobases, since PCR and man-made DNA synthesis become technically challenging and/or costly above this DNA size. Therefore, a convenient, reproducible, and cost-efficient technique to clone large sections of microbial genomes would be highly advantageous.

Frequently bacteria organize genes that work together for a common function as a continuous, physically-linked series across a genome. Large genomic fragments containing many genes that work together for a specific function are very useful for the following reasons: (1) bacteria are able to be engineered for specific purposes in a “quantum leap” using such DNA clones; and (2) basic evolutionary questions are able to be answered using large genomic clones, such as: “Can the cloned gene set be expressed and functional outside of the context of the original genome/species?” These approaches extend the study of genomics by identifying potentially interesting parts of genomes identified via sequencing and studying them in different strain backgrounds. A clear example of this approach is the cloning of protein secretion systems and the subsequent study of these clones (Blondel et al. 2010; Ham et al. 1998; Hansen-Wester, Chakravorty, and Hensel 2004; McDaniel and Kaper 1997; Wilson, Coleman, and Nickerson 2007; Wilson and Nickerson 2006). However, many other gene systems can be studied in this way, with examples including polysaccharide secretion pathways (for capsule and LPS synthesis) and metabolic pathways (anabolism and/or catabolism of key molecules, such as those used in bioremediation). Our ability to extend genomics beyond sequencing to the

utilization of newly-identified multi-gene pathways to engineer bacteria will depend upon our ability to clone, manipulate, and transfer large genomic fragments.

A recent strategy that exploits recombineering and conjugation provides a convenient approach to cloning large bacterial genomic fragments (Blondel et al. 2010; Santiago, Quick, and Wilson 2011; Wilson, Figurski, and Nickerson 2004; Wilson and Nickerson 2007). This approach involves insertion of recombinase sites (*e.g.*, FRT, *loxP*) at positions flanking a targeted genomic region, followed by subsequent recombinase-mediated excision of the region as a non-replicating circular molecule (Fig. 1). Then the excised region is “captured” via either site-specific or homologous recombination onto a conjugative plasmid (such as the broad-host-range IncP plasmid R995) that allows the transfer and isolation of the desired construct in a fresh recipient strain (Fig. 1). The advantages of this approach are (1) the highly specific targeting of exact cloning endpoints using recombineering and (2) the use of conjugation to allow the desired construct to be isolated away from the donor strain (in which the recombination events take place). In addition, except for the synthesis of recombineering PCR products, this protocol takes place entirely in bacterial cells, using basic, low-cost microbiological techniques. Though early approaches used subcloned DNA fragments to allow homologous recombination, the use of recombineering for both the introduction of target flanking sites and the capture on R995 alleviates the need for this subcloning.

2. Targeted cloning of large bacterial genomic fragments

2.1 The VEX-Capture technique

The original technique using this approach is termed VEX-Capture (Wilson, Coleman, and Nickerson 2007; Wilson, Figurski, and Nickerson 2004; Wilson and Nickerson 2006, 2006, 2007). The pVEX series of suicide plasmids was used to introduce *loxP* sites into regions flanking targeted genomic regions via homologous recombination (Fig. 2) (Ayres et al. 1993). Cre recombinase (expressed from a plasmid) was used to excise the targeted region and homologous recombination was used to capture the excised circle (Fig. 2). Note that the homologous recombination is driven by the endogenous bacterial RecA-mediated mechanism. A series of *Salmonella typhimurium* genomic islands ranging from 26 to 50 kilobases in size were targeted for cloning using this technique (Wilson, Coleman, and Nickerson 2007; Wilson, Figurski, and Nickerson 2004; Wilson and Nickerson 2006, 2006). Since these islands contain genes that are unique to *S. typhimurium*, one of the initial basic applications of these clones was to study their gene expression patterns in different bacteria (Wilson, Figurski, and Nickerson 2004; Wilson and Nickerson 2006). Though some *S. typhimurium* genes on the tested genomic island were expressed in all bacteria, several genes displayed genus-specific expression patterns (Fig. 3). This indicated that the mechanisms used to express these genes are absent or function differently in certain bacterial genera. These mechanisms could be the focus of study to understand gene expression functions that work only in certain bacterial groups, such as pathogens or environmental bacteria.

Two separate *S. typhimurium* type III secretion systems were cloned using the VEX-Capture approach (Wilson, Coleman, and Nickerson 2007; Wilson and Nickerson 2006). These systems are encoded at the *Salmonella* pathogenicity island 1 and 2 regions (SPI-1 and SPI-2, respectively) of the *S. typhimurium* genome (McClelland et al. 2001). Both clones are

Fig. 1. General outline of VEX-Capture to clone large genomic fragments. A large fragment of a bacterial genome (generally considered as greater than 10 kilobases) is targeted for excision and cloning by inserting recombinease sites at flanking positions. At least one antibiotic marker gene is required to be associated with the target DNA for subsequent selection. The self-transmissible IncP plasmid R995 serves as a cloning vector that will capture the excised genomic fragment using either a small region of DNA homologous to the excised fragment or a corresponding recombinease site. Also co-resident in the same cell is a plasmid expressing the recombinase that recognizes the recombinease sites. Expression of the recombinase results in excision of the target DNA as a non-replicating circular molecule. This circular molecule will be inserted into R995 via homologous recombination or via the recombinase activity. This construct is conveniently isolated away from the target strain via conjugation to a differentially-marked recipient strain and selection for the appropriate markers. In the recipient strain, structural confirmation of the construct and testing for gene expression and function can occur. In addition, transfer to new bacterial recipients can be performed.

functional and serve to complement protein secretion defects in *S. typhimurium* mutants that are deleted for each SPI-1 and SPI-2 island (Fig. 4). However, the authors found remarkably different results between R995 + SPI-1 and R995 + SPI-2 when tested for expression in other Gram-negative bacteria (Fig. 5). The R995 + SPI-2 clone readily displays expression of SPI-2 (indicated using Western blot analysis of the SseB protein) in other Gram-negative genera, while the R995 + SPI-1 clone displays an expression defect outside of *S. typhimurium* (assayed using Western blot analysis of the SipA and SipC proteins). This result suggests that the regulatory mechanisms controlling SPI-1 and SPI-2 expression have evolved differently and in such a way that manifests itself upon transfer to new bacterial backgrounds.

2.2 VEX-Capture modified

A modification of VEX-Capture was used to clone the type VI secretion system encoded at *Salmonella* pathogenicity island 19 (SPI-19) in the *S. gallinarum* genome (Blondel et al. 2010). In this approach, the *loxP* sites and markers (for chloramphenicol and spectinomycin resistance) were PCR-amplified from the pVEX vectors and inserted into flanking positions using phage λ Red recombination (Fig. 6). The SPI-19 region was excised via Cre recombinase and captured onto R995 using homologous recombination (Fig. 6). The resulting R995 + SPI-19 clone was used to complement the colonization defect of the *S. gallinarum* SPI-19 deletion strain in a chicken infection model (Blondel et al. 2010). In addition, the authors transferred the R995 + SPI-19 clone into *S. enteritidis*, a species that contains significant sequence deviation in SPI-19 relative to *S. gallinarum*, to test if the presence of the *S. gallinarum* SPI-19 would increase *S. enteritidis* chicken colonization.

Interestingly, the presence of SPI-19 decreased the ability of *S. enteritidis* to colonize in this infection model (Blondel et al. 2010). This is consistent with the observations described above that demonstrate genomic island phenotypes can differ greatly, depending on the bacterial background.

2.3 New R995 derivatives allow an “all recombinase” approach

Recently an entirely recombinase-based approach for this technique has been described using modified R995 plasmids (Santiago, Quick, and Wilson 2011). The new series of R995 derivatives encode a range of different marker combinations to increase utility in situations where several markers are used or are already present in the strain background. In addition, these R995 derivatives contain FRT sites that can facilitate the capture of genomic regions that have been excised using the FLP/FRT system (Fig. 7). A major advantage to this approach is that no regions of homology are needed to be cloned into any plasmids. Thus, the only step that takes place outside of cells is the amplification of the PCR products used for λ Red insertion of FRT sites into the flanking positions in the genome. This technique was demonstrated by cloning 20-kilobase regions from the *S. typhimurium* and *Escherichia coli* genomes (Santiago, Quick, and Wilson 2011).

2.4 Catalogue of reagents

Table 1 serves as a summary list of reagents used for the recombinase/conjugation-based cloning of genomic fragments. The PCR template plasmids are suicide plasmids and can

Fig. 2. The VEX-Capture system. Excision and capture of a section of the *S. typhimurium* genome is depicted to illustrate the functioning of the VEX-Capture system. In step one, differentially-marked pVEX vectors containing DNA fragments homologous to the ends of the targeted genomic region are integrated at the desired locations to form a double

cointegrate. In this structure, single *loxP* sites are located on either side of the targeted region. In step two, the targeted region is excised from the genome by the Cre recombinase, and the excised circle is “captured” via homologous recombination with the R995 VC plasmid. Note that the capture fragment on R995 VC is shown as targeted to one end of the excised genomic region, but it can be targeted to any location on the excised region. In step 3, the R995 VC-excised circle plasmid is transferred to an *E. coli* recipient to create a strain containing the captured genomic fragment. Diagram not drawn to scale. Reprinted from (Wilson and Nickerson 2007).

Fig. 3. RT-PCR analysis of *S. typhimurium* island 4305 after transfer to different Gram-negative hosts. The indicated Gram-negative strains containing R995 + *S. typhimurium* island 4305 were analyzed for expression of island genes STM4305, STM4315, STM4319 and the R995 replication gene *trfA* (which serves a positive control). Total RNA from each strain was isolated and reversed transcribed, and the samples were PCR-amplified using primers against the indicated genes. The (+) and (-) lanes indicate samples with and without the reverse transcriptase step, respectively, and the (D) lane indicates where R995 + island 4305 DNA isolated from each was used as template. PCR samples were run on agarose gels and stained with ethidium bromide. The boxed pictures indicate where expression of the gene is not detectable. This figure demonstrates genus-specific expression patterns for those island genes. Reprinted from (Wilson and Nickerson 2006).

Fig. 4. R995 + SPI-1 and R995 + SPI-2 clones complement corresponding *S. typhimurium* SPI-1 and SPI-2 deletion mutants for substrate protein secretion. Panel A: Western blot analysis of protein secretion preparations and total cell lysates from *S. typhimurium* delta SPI-1 strains containing either R995, R995 + SPI-1, or R995 + SPI-1 *invA* plasmids. The last plasmid contains a mutation in the *invA* gene encoding a SPI-1 type III system protein that is essential for SPI-1-mediated secretion. Antibodies against the SPI-1 secreted substrate SipC and the non-secreted bacterial cellular protein p20 are used. Panel B: Western blot analysis as in Panel A but using *S. typhimurium* delta SPI-2 strains containing either R995, R995 + SPI-2, or R995 + SPI-2 *ssaV* (mutation for the *ssaV* gene essential for SPI-2 secretion activity). Antibodies against the SPI-2 protein substrate SseB are used. The results of both panels demonstrate that the cloned SPI-1 and SPI-2 regions on R995 are functional and complement deleted SPI-1 and SPI-2 secretion systems. Reprinted from (Wilson, Coleman, and Nickerson 2007; Wilson and Nickerson 2006).

Fig. 5. Different expression patterns for SPI-1 and SPI-2 in different Gram-negative bacterial genera. Panel A: Plasmid R995 + SPI-1 was analyzed for expression of the SPI-1 protein SipC via Western blot analysis in *S. typhimurium*, *E. coli*, and *Pseudomonas putida*. In addition, the samples were also probed for the bacterial housekeeping p20 protein and the R995-encoded protein KleA as controls. The samples shown are total cell lysates of each strain. SipC expression is not detectable in *E. coli*, *P. putida*, attenuated in *P. aeruginosa* and *Agrobacterium tumefaciens* (the last two species not shown). Panel B: Plasmid R995 + SPI-2 expression was analyzed via Western blot assay against the SPI-2 protein SseB in various Gram-negative

bacteria. In contrast to SPI-1, expression of SPI-2 was readily detected in a range of bacterial backgrounds. Two points are of particular note: (1) In *S. typhimurium*, SPI-2 expression is regulated by growth media conditions, such that 10 mM MgCl₂ and pH 7.5 repress expression (MgM 10 media) and 8 μ M MgCl₂ and pH 5.5 activate expression (MgM 8 media). However, expression from R995 + SPI-2 does not follow this regulation, except in the *E. coli* strain TOP10. R995 + SPI-1 expression shows a similar result in *S. typhimurium* in relation to its regulation by sodium chloride; and (2) *P. putida* appears to be recalcitrant to both SPI-1 and SPI-2 expression. Reprinted from (Wilson, Coleman, and Nickerson 2007; Wilson and Nickerson 2006).

Fig. 6. Schematic representation of the capture of SPI-19 from *S. gallinarum* 287/91 using a modified VEX-Capture method. To clone the type VI secretion system from the *S. gallinarum* genome, Blondel *et. al.* PCR-amplified markers and *loxP* sites from pVEX vectors and inserted them into flanking positions using phage λ Red recombination. The Cre-excised circular molecule was captured by R995 via homologous recombination, and the construct was isolated upon conjugation to an *E. coli* recipient. This construct was used for complementation analysis in a chicken model of infection using *S. gallinarum* and *S. enteritidis* strains and demonstrates the utility of R995 capture plasmids for *in vivo* pathogenesis studies. Reprinted from (Blondel *et al.* 2010).

Fig. 7. An “all recombinase” approach to cloning large genomic DNA fragments to R995. This procedure utilizes specially designed R995 derivatives containing FRT sites that can be used as insertion points for a genomic fragment excised using the Flp/FRT system. A targeted DNA region in a bacterial genome is flanked by FRT sites and an antibiotic resistance marker as diagrammed using λ Red recombination. To accomplish this, the “unmarked” FRT site (to the left of the target DNA in the chromosome) is introduced via standard λ Red recombination markers (in Table 1) followed by Flp-mediated deletion of the marker to leave the single, unmarked FRT site. Next, the second flanking FRT site is introduced using a PCR fragment designed with a marker and single FRT site, such that the marker is located between the FRT site and the target DNA. In this example, the marker encodes kanamycin resistance. An R995 derivative containing an FRT site (and encoding tetracycline resistance in this example) is transferred to this strain via conjugation, and then the Flp-expressing plasmid pCP20 is introduced via electroporation. The electroporation outgrowth culture can be used directly as a donor for conjugation with a rifampicin (Rif)-resistant recipient strain. Alternatively, the electroporation can be plated on media containing tetracycline (Tc) and kanamycin (Km) and the colonies can be used as donor. The conjugation is plated on media containing Rif, Tc, and Km to select recipients that have obtained the cloned target DNA on R995. The transconjugants can be used to confirm the clone. A transconjugant can also be used as a donor for transfer of the clone to other bacterial strains for subsequent studies. Reprinted from (Santiago, Quick, and Wilson 2011).

<u>Plasmid or strain</u>	<u>Characteristics</u>	<u>Reference</u>
<u>Template plasmids</u>		
pKD3	FRT:Km-r:FRT, R6K ori	Datsenko, 2000
pKD4	FRT:Km-r:FRT, R6K ori	Datsenko, 2000
pJW101	FRT:Tp-r:FRT, R6K ori	Quick, 2010
pJW102	FRT:Sp-r:FRT, R6K ori	Quick, 2010
pJW107	FRT:Tc-r:FRT, R6K ori	This chapter
pVEX1212	<i>loxP</i> :Sp-r, P1 ori	Ayres, 1993
pVEX2212	<i>loxP</i> :Cm-r, R6K ori	Ayres, 1993
<u>Lambda Red plasmids</u>		
pKD46	Ap-r	Datsenko, 2000
pJW103	Km-r	Quick, 2010
pJW104	Cm-r	Quick, 2010
pJW105	Tp-r	Quick, 2010
pJW106	Sp-r	Quick, 2010
<u>Recombinase plasmids</u>		
pCP20	Flp, Ap-r, Cm-r	Datsenko, 2000
pEKA30	Cre, Ap-r, Tc-r	Wilson, 2004
pEKA16	IPTG-inducible Cre, Ap-r, Tc-r	Wilson, 2004
<u>R995 derivatives</u>		
R995	Km-r, Tc-r	Pansegrau, 1994
R995 Km Cm	$\Delta tetRA::FRT$ -Cm-FRT	Santiago, 2011
R995 Km Tp	$\Delta tetRA::FRT$ -Tp-FRT	Santiago, 2011
R995 Km Sp	$\Delta tetRA::FRT$ -Sp-FRT	Santiago, 2011
R995 Tc Cm	$\Delta aphA::FRT$ -Cm-FRT	Santiago, 2011
R995 Tc Tp	$\Delta aphA::FRT$ -Cm-FRT	Santiago, 2011
R995 Tc Sp	$\Delta aphA::FRT$ -Cm-FRT	Santiago, 2011
R995 Km	$\Delta tetRA::FRT$	Santiago, 2011
R995 Tc	$\Delta aphA::FRT$	Santiago, 2011
R995 Cm	$\Delta tetRA::FRT$, $\Delta aphA::Cm-r$	Santiago, 2011
R995 Tp	$\Delta tetRA::FRT$, $\Delta aphA::Tp-r$	Santiago, 2011
R995 Sp	$\Delta tetRA::FRT$, $\Delta aphA::Sp-r$	Santiago, 2011
<u>Strains</u>		
AS11	<i>pir</i> ⁺ (for R6K ori plasmids)	Ayres, 1993
EKA260	<i>repA</i> ⁺ (for P1 ori plasmids)	Ayres, 1993
TOP10 Rif	Rif-r recipient	Wilson, 2004

Table 1. Catalogue of reagents for recombinase/conjugation cloning. Please note that the template plasmids are suicide plasmids that require either AS11 or EKA260 for replication and that the λ Red plasmids and pCP20 are temperature-sensitive for replication (requiring 30 degrees C). The pJW plasmids are derived from either pKD3 (pJW101 and pJW102) or pKD46 (pJW103, pJW104, pJW105, and pJW106) (Quick, Shah, and Wilson 2010).

only replicate in corresponding strains that encode either the R6K Pir protein or P1 RepA protein (Ayres et al. 1993; Datsenko and Wanner 2000). This allows the PCR reaction to be directly electroporated into target cells with no background problems caused by the replication of the templates. It is worthwhile to note the PCR template plasmids with FRT sites contain two such sites flanking a given antibiotic resistance marker. Thus, care must be taken to amplify products containing only one FRT site for the second flanking insertion into the genome to avoid marker loss problems upon FLP expression (please refer to Fig. 7 for more details). It is also worthwhile to note that the self-transmissible IncP plasmid R995 displays a remarkably broad-host-range for both its conjugation and replication system (Adamczyk and Jagura-Burdzy 2003; Pansegrau et al. 1994; Thorsted et al. 1998). This facilitates R995 conjugative transfer to a wide variety of Gram-negative and Gram-positive bacteria and replication in almost all Gram-negative bacteria. Any other conjugative plasmid could be used for this procedure. However, IncP plasmid R995 and related plasmids are excellent options due to their broad-host-range, fully sequenced genomes, and high degree of characterization (especially for the IncPα plasmids R995, RK2, RP4, etc.).

3. Conclusion

Recombineering and conjugation can be exploited to provide a convenient, reproducible, and cost-effective technique for cloning large bacterial genomic fragments. This technique can be performed using easily obtained PCR products, readily available plasmids and strains, and simple, basic microbiology protocols. One question regarding the use of this system is: how large a genomic fragment can be accommodated by R995? So far, the biggest fragment cloned using this technique has been about 50 kilobases, but the upper limits of size have not yet been tested in any systematic way. To make genomic clones more amenable to medical or environmental applications, removal of antibiotic resistance markers and the conjugative transfer system would need to be accomplished. We are currently pursuing the development of alternative selection schemes and removable conjugation systems to address this issue. Overall, the use of the recombinase/conjugation cloning approach is currently underdeveloped as a technique and could expand the field of genomics by providing experiment-based strategies to answer important evolutionary questions.

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