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Jeremy R. Haag
Washington University

Dong W. Lee
Texas A&M University

Rodolfo Aramayo
Texas A&M University

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Abstract

We report the construction of a Destination Vector, called pJHAM007, for the targeted integration of DNA sequences at the *histidine-3* (*his-3*) locus of *Neurospora crassa*. pJHAM007 has all the necessary features required to perform a simple, rapid and efficient GATEWAY™ recombinational cloning with an Entry Clone to yield a *his-3*-gene replacement Destination Vector.

A GATEWAY™ Destination Vector For High-Throughput Construction of *Neurospora crassa histidine-3* Gene Replacement Plasmids

Haag, Jeremy R.[1], Lee, Dong, W.[2], and Aramayo, Rodolfo[2,3]. [1]Department of Biology, Washington University, Campus Box 1137, 1 Brookings Drive, St. Louis, MO 63130. [2]Department of Biology, Texas A&M University, Room 415, Building BSBW, College Station, TX 77843-3258

We report the construction of a Destination Vector, called pJHAM007, for the targeted integration of DNA sequences at the *histidine-3* (*his-3*) locus of *Neurospora crassa*. pJHAM007 has all the necessary features required to perform a simple, rapid and efficient GATEWAY™ recombinational cloning with an Entry Clone to yield a *his-3*-gene replacement Destination Vector. Fungal Genet Newsl 50:6-8

Gene replacement is a powerful tool to construct isogenic strains containing different DNA sequences integrated at the same chromosomal position. The most popular locus used for gene targeting in *Neurospora crassa* is of the metabolic gene *histidine-3* (*his-3*). Several generations of plasmids for integration at this chromosomal position have been constructed (Sachs and Ebbole 1990 Fungal Genet. Newsl. **37**: 35-36, Ebbole 1990 Fungal Genet. Newsl. **37**: 15, Margolin*, et al.* 1997 Fungal Genet. Newsl. **44**: 34-36, Aramayo and Metzenberg 1996 Fungal Genet. Newsl. **43**: 9-13). Recently, we described the construction of a new set of *N. crassa* strains and plasmids that represent a significant improvement over previous systems because they allow the investigator to screen in one simple step for homokaryotic transformants containing the insertion of a test sequence among a population of primary histidine-independent transformants (Lee*, et al.* 2003 Curr. Genet. DOI 10.1007/s00294-002-0366-z). These new tools have significantly reduced the time it takes to construct new *N. crassa* strains. To expedite this system even further, we have designed and constructed a new plasmid, pJHAM007, that can be used for the high-throughput cloning of DNA inserts, to generate *his-3*-gene replacement plasmids for different types of large- or small-scale genome analysis.

Plasmid pJHAM007 is based on the GATEWAY™ system (Walhout*, et al.* 2000 Method. Enzymol. **328**: 575-592). GATEWAY™ is a novel universal system for cloning and subcloning DNA sequences that uses phage lambda (λ)-based site-specific recombination (Landy 1989 Annu. Rev. Biochem. **58**: 913-949). This Recombinational Cloning (RC) consists on two reactions: (1) The LR Reaction (*att*L X *att*R → *att*B + *att*P), mediated by the Integrase (Int), Integration Host Factor (IHF) and excisionase (Xis); and (2) the BP Reaction (*att*B X *att*P → *att*L + *att*R), mediated by the Int and IHF proteins. By providing different combinations of the recombination proteins and sites, the direction of the reaction can be easily controlled (Walhout*, et al.* 2000 Method. Enzymol. **328**: 575-592, Hartley*, et al.* 2000 Genome Res. **10**: 1788-1795).

Escherichia coli DB3.1 and DH5α (Invitrogen, Carlsbad, CA, USA) were the hosts for bacterial manipulations. When non-methylated DNA was needed for enzyme digestions, either GM2163--an *E. coli* K12 derivative containing, among others markers, *dam13::Tn9* (Cam^R) and *dcm-6* mutations (New England BioLabs (NEB), Beverly, MA, USA), or JM110--an *E. coli* K12 derivative containing, among others, *dam* and *dcm* mutations (Yanisch-Perron*, et al.* 1985 Gene **33**: 103-119) was used. *E. coli* DB3.1 was routinely used to propagate plasmids. In contrast, *E. coli* DH5α was used only to propagate the plasmid products of the BP and LR reactions. It is important to understand that Destination Vectors carrying the *ccd*B gene cannot propagate in *E. coli* DH5α and most *E. coli* strains, because the CcdB protein, a natural analogue of the quinolone antibiotics (*e.g.,* ciprofloxacin, enoxacin, etc.), binds to the DNA *gyrase subunit A*, the product of the *gyrA* gene, turning it into a cellular poison (Bahassi*, et al.* 1999 J. Biol. Chem. **274**: 10936-10944).
E. coli strains DB3.1 and DH5α were routinely grown in LB liquid culture, or on LB agar plates (15 g/l) containing the following antibiotics, as indicated in the text: ampicillin (Amp), 150 μg/ml; chloramphenicol (Cm), 30 μg/ml; kanamycin (Km), 50 μg/ml.

Most DNA manipulations were done following standard procedures as described (Sambrook*, et al.* 1989, Ausubel*, et al.* 1987, Pratt and Aramayo 2002 Fungal Genet. Biol. **37**: 56-71). For DNA sequencing we used the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (PEBiosystems, Foster City, CA, USA). Sequences were generated on an Applied Biosystems Model 377 or 373 automated DNA sequencer at GeneTechnologies Laboratory (Institute of Developmental and Molecular Biology—IDMB, Texas A&M University, College Station, TX, USA).

PCR reactions were performed in 50 μl reactions for 40 cycles (94°C, 30 s; 60.8°C, 30 s; 68°C, 9.5 min) with the Clontech Advantage 2 PCR System (BD Biosciences Clontech, Palo Alto, CA, USA) using the manufacturer's specifications.

To amplify the 9,001 bp chromosomal DNA region containing the *N. crassa* NCU02764.1 gene, we used two primers: OJHAM013 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTcgcgaacgtagaaggattattaggcaaagt-3') and OJHAM014 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTgtcagtcagtcagtcagtcaaccagt-3'). The 29 upper-case characters correspond to the *att*B1 and *att*B2 primer sequences present in OJHAM013 and OJHAM014, respectively. The 30 lower-case characters correspond to the priming sites of the oligonucleotides in the *N. crassa* chromosomal region.

After PCR, the resulting PCR products were purified from *att*B primers and *att*B primer-dimers by either using PEG precipitation (30% (w/v) PEG 8000/30 mM $MgCl_2$, as recommended by Invitrogen), or extraction from a 1% (w/v) agarose gel and purification with the Wizard PCR Preps DNA Purification Resin as recommended by Promega (Promega, Madison, WI, USA).

The LR and the BP GATEWAY™ reactions were both performed as recommended by the manufacturer (Invitrogen).

Results and Discussion

Construction of pJHAM007, a GATEWAY™-compatible *his-3*-gene replacement Destination Vector. To demonstrate the feasibility of using GATEWAY™ to direct the integration of constructs at the *his-3* locus of *N. crassa*, we started by converting the *his-3*-gene replacement vector pJHAM003 (Lee*, et al.* 2003 Curr. Genet. DOI 10.1007/s00294-002-0366-z) into a Destination Vector called pJHAM007. The *his-3*-gene replacement plasmid, pJHAM007, was constructed by inserting a 2 kb *Bgl*II-*Eco*RV cassette removed from pDEST14 (Invitrogen) and containing the *att*R1, the *cat*$^{+}$ = *chloramphenicol acetyl transferase* gene (chloramphenicol resistance), the *ccd*B gene and the *att*R2, into the *Bam*HI-*Pme*I restriction sites of pJHAM003 (Figure 1). We confirmed the construction of this plasmid by digesting pJHAM007 using several different restriction enzymes. Following a similar strategy, gene-replacement vectors for other loci can be converted into GATEWAY™ Destination Vectors.

RC-mediated Cloning of PCR Products To Generate Entry Clones. Complementation of a mutant phenotype by an ectopically integrated DNA fragment is a common way to demonstrate gene function. Because filamentous fungi can have large genes and complex promoters, it is not uncommon for those DNA fragments to be large, in order for them to contain all the necessary regulatory elements.

To direct the integration of a DNA fragment at the *his-3* locus using the *his-3*-gene replacement Destination Vector pJHAM007, the PCR fragment first needs to be present in an Entry Clone. It is known that the efficiency of the *in vitro* RC reactions decreases with increasing size of the DNAs involved (Hartley*, et al.* 2000 Genome Res. **10**: 1788-1795), but given the predicted need to occasionally use large DNA fragments, we first decided to test the limits of the RC-cloning reaction.

We therefore designed PCR primers containing *att*B sites to amplify a 9 kb DNA region from Linkage Group I (LG I) containing the *N. crassa* NCU02764.1 gene. PCR products flanked by *att*B sites can be generated by incorporating *att*B sites (25 base + 4 G residues) at the 5'-end of PCR primers (*att*B1 in the forward primer and *att*B2 in the reverse primer) and cloned by BP Recombination, into *att*P-containing vectors in the presence of Int and IHF (BP Clonase) to generate Entry Clones capable of recombining with Destination Vectors (Hartley*, et al.* 2000 Genome Res. **10**: 1788-1795). For this, 500 ng of the 9 kb PCR product were mixed with 300 ng of linearized pDONR201 plasmid (Invitrogen) and BP Clonase following the manufacturer's recommendations (Invitrogen). Following transformation into *E. coli* DH5α cells and *Kanamycin-Resistance* (Km^R) selection, we analyzed 24 positive clones. Of those, only five were determined to be correct. We selected one correct clone and named it pJHAM009. The resulting plasmid, pJHAM009, is a GATEWAY™-compatible Entry Clone and its insert can therefore be transferred to any other Destination Vector with great versatility and efficiency.

RC-mediated Cloning of the insert from the Entry Clone (pJHAM009), into the *his-3*-Gene Replacement Destination Vector (pJHAM007), to generate the *his-3*-Gene Replacement plasmid (pJHAM010). The next step in the construction of a *his-3*-gene replacement Destination Vector is the transfer of the DNA insert present in the Entry Clone to the Destination Vector. This reaction is catalyzed by the LR Clonase, a mixture of the Int, IHF, and Xis proteins. For this, 140 ng of pJHAM009 Entry Clone were mixed with 300 ng of linearized pJHAM007 plasmid and LR Clonase following the manufacturer's recommendations (Invitrogen). Following transformation into *E. coli* DH5α cells and *Ampicillin-Resistance* (Ap^R) selection, we analyzed 24 clones, among thousands of transformants. All 24 clones were determined to contain the expected insert size and all had the predicted restriction pattern. We selected one positive clone and named it pJHAM010. These results demonstrate that once in an Entry Clone, inserts as large as 9 kb can be transferred to any other Destination Vector with great versatility and efficiency, especially considering the large size of the resulting plasmid (in this case 21 kb).

Use of plasmid pJHAM007-derivative (pJHAM010) to direct the integration of DNA inserts at the *his-3* chromosomal position. To demonstrate the feasibility of using gene replacement plasmids derived from pJHAM007 to direct the integration of DNA inserts at the *his-3* chromosomal position, we transformed strains DLNCT62A and DLNCR83A (Lee*, et al.* 2003 Curr. Genet. DOI 10.1007/s00294-002-0366-z), each using 50 μg of *Sfi*I-linearized plasmid pJHAM010. We selected 12 colonies from each transformation and tested them for FUDR (2'-deoxy-5-fluorouridine or (+)-5-fluorodeoxyuridine, filter sterilized) and hygromycin-resistance/sensitivity as described in (Lee*, et al.* 2003 Curr. Genet. DOI 10.1007/s00294-002-0366-z). Six FUDR-resistant, hygromycin-sensitive transformants obtained from DLNCT62A and seven obtained from DLNCR83A, were selected for further analysis. DNA was extracted from one DLNCT62A- and one DLNCR83A-derived transformant and Southern blot hybridization analysis was used to determine if a true gene replacement event had occurred in these tranformants. As predicted, both transformants contained the insert present in plasmid pJHAM010 integrated at the *his-3* locus (data not shown).

In summary, a large PCR fragment obtained from *N. crassa* genomic DNA was cloned into a GATEWAY™-compatible Entry Vector, transferred into a *his-3*-gene replacement Destination Vector and integrated back at the *N. crassa his-3* chromosomal locus with minimal work and in a short period of time. We predict that the use of this technology will accelerate the development of our understanding of *N. crassa* biology considerably.

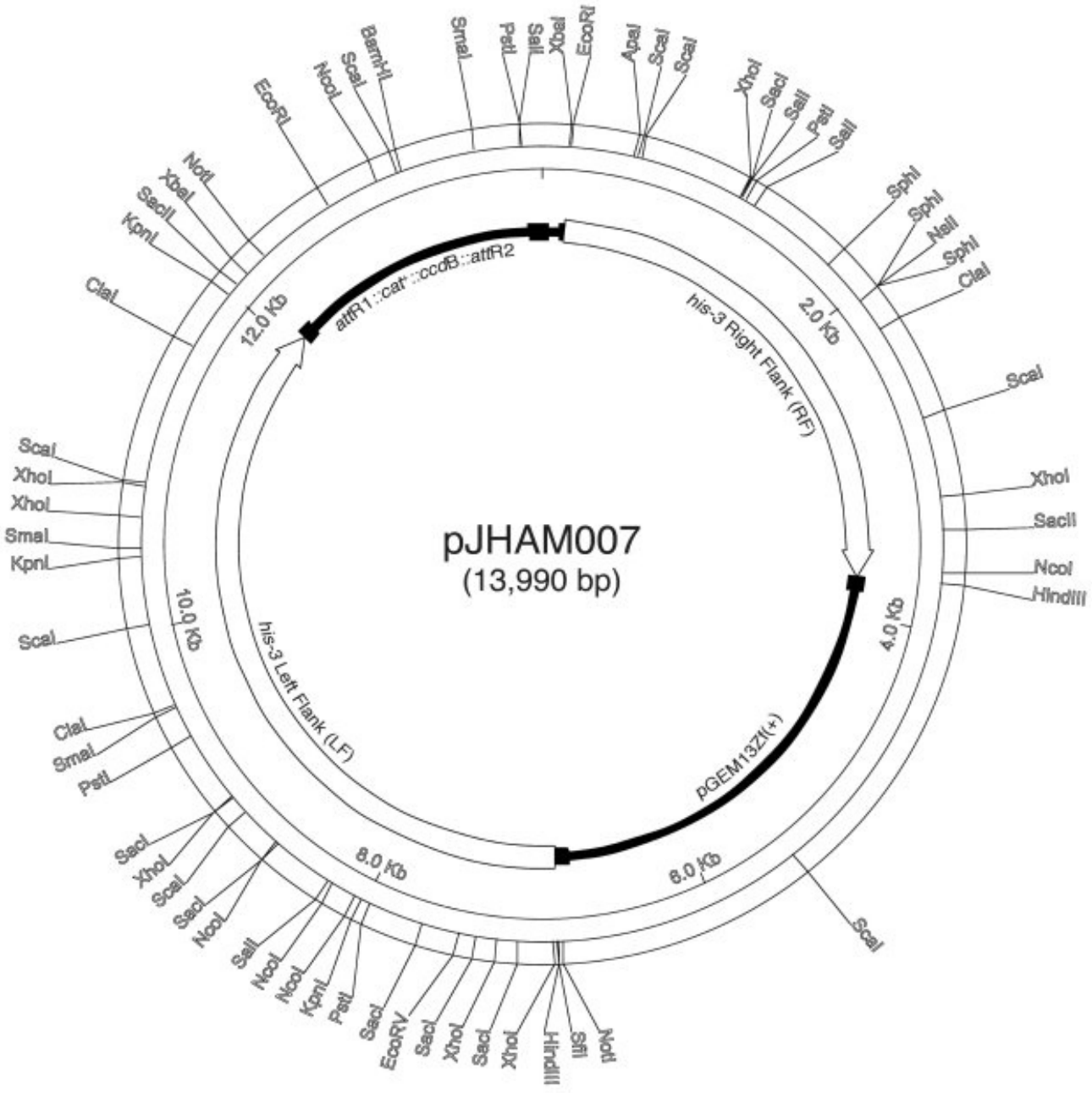

Figure 1. pJHAM007, a *his-3*-gene replacement GATEWAY™-compatible Destination Vector. Circular representation of plasmid pJHAM007 is presented along with the main restriction sites. PGEM13Zf(+) = vector containing a ColE1 origin of replication (Promega), *att*R1 and *att*R2 = attachment sites, *cat*$^{+}$ = *chloramphenicol acetyl transferase* gene (chloramphenicol resistance), *ccd*B = CcdB protein, a quinolone-like antibiotic protein. The left and the right flanks correspond to DNA sequences that flank the site of insertion located downstream of the *his-3* gene. The GeneBank Accession number of pJHAM007 is AF541939.

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