research articles Journal of proteome research

# High-Throughput Cloning of *Campylobacter jejuni* ORFs by in Vivo Recombination in *Escherichia coli*

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**Received November 26, 2003**

A rate-limiting and costly step in many proteomics analyses is the cloning of all of the ORFs for an organism into technique-specific vectors. Here, we describe the generation of a *Campylobacter jejuni* expression clone set using a high-throughput cloning approach based on recombination in *E. coli*. The approach uses native *E. coli* recombination functions and requires no in vitro enzymatic steps or special strains. Our results indicate that this approach is an efficient and economical alternative for high-throughput cloning.

**Keywords:** high-throughput cloning • Campylobacter • proteomics

## Introduction

Understanding protein and pathway function at a system-wide level requires proteomic techniques that analyze hundreds of proteins in parallel. A common rate-limiting step in many proteome-scale analyses is the cloning of an organism's predicted open reading frames (ORFs) into technique-specific vectors. For example, yeast two-hybrid screens, protein microarray assays, systematic protein complex identification, and protein structure determinations, when applied at the proteome-scale, require large clone sets for expressing proteins as fusions to different functional moieties. Moreover, a thorough understanding of an organism's proteome will require the application of multiple techniques, and therefore, the need to subclone the same set of ORFs into several different vectors. Thus, the development of high-throughput cloning strategies that are fast, efficient, and economical is of paramount importance to proteomic studies.

Cloning methods to date have fallen into two categories, those that require in vitro reactions and those that use in vivo recombination functions. Some in vitro strategies such as the Cre/*lox*[1] and Gateway[2,3] methods use purified recombinases to mediate site-specific recombination between an insert and a vector. Another in vitro method, ligation-independent cloning (LIC), takes advantage of the 3′ to 5′ exonuclease activity of certain DNA polymerases (e.g., T4, Pfu) to generate small single-stranded overhangs on PCR-generated inserts, which can then anneal to similarly generated overhangs in the cloning vector.[4,5] Although both the in vitro recombination and LIC methods are more efficient than traditional methods using restriction enzymes and ligases, they still require transformation of *E. coli* to recover the recombinant clones, and the additional time and expense of the in vitro enzymatic steps.

A potentially faster and more economical approach would be to utilize the in vivo homologous recombination functions of a host strain, ideally *E. coli*, to insert PCR-amplified ORFs into vectors without in vitro steps. Perhaps the simplest manifestation of such a method is to co-transform *E. coli* with a linearized vector and a DNA fragment containing an ORF flanked with sequences identical to the sequences flanking the cloning site in the vector.[6,7] Homologous recombination between the identical sequences at the ends of the insert and the vector results in directional insertion of the ORF into the vector. Although clearly promising, this method has not been used for high-throughput cloning, perhaps because of the low efficiency reported.[6,7] A similar in vivo approach has been described in the yeast *Saccharomyces cerevisiae* where efficient homologous recombination facilitates high-throughput cloning into yeast vectors.[8,9] Recombinational cloning in yeast has not become a generally useful strategy, however, in part because it requires that the vectors contain yeast markers and replication origins. For many applications, it would be more beneficial to employ recombination in *E. coli* rather than yeast, as *E. coli* grows faster and most vector sets are designed for propagation in *E. coli*.

In this study, we have optimized the *E. coli* in vivo recombinational cloning method and used it to efficiently generate a proteome-scale expression clone set for the bacterial pathogen *Campylobacter jejuni*, a leading cause of food-borne diarrheal disease in humans.[10] This clone set enables expression and purification of GST-His-tagged *C. jejuni* proteins that could be used for a variety of functional studies. Our results demonstrate the usefulness of the *E. coli* in vivo recombinational

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10.1021/pr0341134 CCC: $27.50 
Published on Web 03/30/2004

cloning method for the high-throughput generation of clone sets for proteome analyses.

## Experimental Section

**Microbial Strains and Plasmids.** The bacterial strains used included *E. coli* KC8 (*pyrF*::Tn5 *hsdR leuB600 trpC9830 lac*Δ74 *strA gal*K *hisB436*),[11] *E. coli* SURE (Stratagene, La Jolla, CA), *E. coli* BL21(DE3) (Stratagene, La Jolla, CA), *E. coli* BUN10 (*recBC, sbcA*),[1] and *E. coli* MG1655 (CGSC: *E. coli* Genetic Stock Center). Strains containing pTLJ03-derived clones were grown in LB containing 100 μg/mL ampicillin unless otherwise specified. Plasmid maps, details, and sequences are available at http://proteome.wayne.edu. pTLJ03, an expression vector that generates N-terminal GST-His-tagged fusion proteins, was constructed by inserting a PCR product encoding GST-EK-His and containing the 5RT1-*Eco*R1-*Bam*H1-3RT1 sequence into pET-11a (Novagen, Madison, WI). The GST-EK-6xHis region was amplified from pTLJ02 (Supporting Information) with a 5′ primer, (5′TAA CTT TAA GAA GGA GAT ATA CAT ATG TCC CCT ATA CTA GGT TAT 3′) that included a 24 bp tag matching pET-11a, and a 3′ primer, (5′AAT TCC GGC GAT ACA GTC AAG TGA TGG TGA TGG TGA TGG T 3′) that contained a 20 bp tag matching the 5RT1 sequence. The product was PCR ligated to an oligo (5′*TTG ACT GTA TCG CCG GAA TTC* GGG CCC GGA TCC *CTG CAG CCA AGC TAA TTC CGG*3′) that included the "5RT1-*Eco*R1-*Bam*H1-3RT1" sequence (the 5RT1 and 3RT1 sequences are italicized). The forward primer, 5′TAA CTT TAA GAA GGA GAT ATA CA3′, matched the pET-11a sequence and the reverse primer, 5′ TAT CAC GAG GCC CTT TCG TCT TCA AGC CGG AAT TAG CTT GGC TGC A 3′, matched the 3RT1 and was tagged with sequence (26 bp) matching pET-11a. Recombinational cloning (as described below) was used to insert the pET-11a-tagged PCR product into pET-11a, which had been linearized using *Nde*I and *BamH*I. Recombination sites were verified by sequencing.

**PCR Amplification.** There were 1685 *C. jejuni* ORFs targeted for amplification including 1654 ORFs that were predicted by the Sanger Center[12] together with 31 more ORFs predicted by TIGR (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). Gene-specific primers were designed using the program Primer 3 (S. Rozen, H. J. Skaletsky, http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and Perl scripts written by Dan Liu (CMMG Bioinformatics Facility, Wayne State University). The 21 bp recombination tags 5RT1 (5′TTG ACT GTA TCG CCG GAA TTC3′) and 3RT1 (5′CCG GAA TTA GCT TGG CTG CAG3′) were added to the ends of the 5′ and 3′ gene-specific primers, respectively. Oligos were synthesized by IDT (Coralville, IA). Genomic DNA from *C. jejuni* strain NCTC11168 served as a template for PCR and was a gift from Victor DiRita (University of Michigan, Ann Arbor). The DNA polymerase mixture Herculase (Stratagene, La Jolla, CA) was used as per manufacturers instructions. Secondary PCR amplification was performed using the primary reactions (diluted 1:10) as template. The primers were as follows: forward primer, 5′ACT ACC ATC ACC ATC ACC ATC ACT TGA CTG TAT CGC CGG AAT T3′; reverse primer, 5′CAC GAG GCC CTT TCG TCT TCA AGC CGG AAT TAG CTT GGC TGC3′. Colony PCR was performed to verify the presence of insert within the expression clones using Taq DNA polymerase (Invitrogen, Carlsbad, CA), pTLJ03-specific primers 5′ TAA TAC GAC TCA CTA TAG 3′ and 5′ TAT CAC GAG GCC CTT TCG TCT TCA AGC CGG AAT TAG CTT GGC TGC A 3′, and bacterial colonies as templates.

**Recombinational Cloning.** Chemically competent *E. coli* cells were prepared and transformed using the TSB protocol.[3] Recombinational cloning transformations utilized vector pTLJ03 that had been linearized via restriction enzyme digestion using *Eco*R1 and *Bam*H1, and purified with a Centricon-100 filter (Millipore, Billericca, MA). Low-throughput recombinational cloning was performed in standard 1.5 mL microcentrifuge tubes. To 50 μL of 1× KCM (0.1 M KCL, 30 mM $CaCl_2$, 50 mM $MgCl_2$), 5 μL (∼50 ng) of purified linearized vector DNA, and 5 μL (∼50 ng) of tagged PCR product (insert) were added followed by chemically competent *E. coli* KC8 cells (50 μL). The mixture was incubated on ice (20 min) and then at room temperature (10 min). Liquid LB (500 μL) was added followed by incubation at 37 °C (1.5 h). Prior to plating, cells were pelleted in a microcentrifuge, and resuspended in approximately 100 μL of the supernatant. The entire transformation mix was then plated on one LB plate containing the appropriate antibiotic. High-throughput recombinational cloning was performed as follows. The primary PCR ORF amplification products were precipitated. The PCR products (100 ng in 15 μL) were transferred to skirted 96-well PCR plates (Dot Scientific, Inc., Burton, MI). Precipitation mix (150 μL) containing 91% ethanol and 143 mM sodium acetate, pH 5.2 was added to each well, mixed, and incubated at −80 °C overnight. Plates were then centrifuged at 3500 rpm at 4 °C for 45 min with an Eppendorf Centrifuge 5810 (Eppendorf, Westbury, NY), and the supernatant was removed by inverting the plate. A wash of ice-cold 80% ethanol (150 μL) was added to each well and the plates were centrifuged for 20 min. The wash was then discarded and the samples were dried at room temperature followed by resuspension in 10 μL sterile $H_2O$. Linearized vector (50 ng) and 50 μL of 1X KCM were added to the 96-well plates containing the 10 μL of precipitated PCR products and mixed. *E. coli* KC8 chemically competent cells (50 μL) were added to each well. Plates were sealed and incubated for 30 min on ice-ethanol, followed by 10 min at room temperature. LB medium (80 μL) was added to each well, mixed, and incubated for 2 h at 37°. The transformation mix (150 μL) was plated robotically on 6-well culture plates containing 3.2 mL LB ampicillin. The plates were then incubated overnight at 37 °C. Single colonies were picked back into 96-well plates containing LB ampicillin and incubated overnight (37 °C). Prior to −80 °C storage, a sample (5 μL) of each culture was spotted robotically onto omni plates (Nunc, Rochester, NY) containing LB agar with ampicillin and grown overnight 37 °C. These plates provided the template for colony PCR (see above). The insert-positive clones were isolated using QIAprep 96 Turbo Miniprep Kits (Qiagen, Valencia, CA) and transformed into *E. coli* SURE cells for extended storage. Recombinational cloning using secondary PCR products proceeded as above except that the PCR products were not ethanol precipitated. Instead, they were used without any purification or were used after purification on Sephadex columns.

**Clone Sequencing and Analysis.** Sequencing was conducted by the Genomics Technology Support Facility at Michigan State University (*http://genomics.msu.edu*). Plasmid DNA was prepared from 3 mL *E. coli* SURE cultures on an AutoGen PI-50a Automatic DNA Isolation System (AutoGen, Inc., Holliston, MA). Sequencing was performed using Applied Biosystems 3730xl DNA Analyzers (Applied Biosystems, Foster City, CA) and the primer 5′GCT ATC CCA CAA ATT GAT AA 3′. Sequence analysis to validate the identity and fidelity of the inserts cloned was facilitated using a series of Perl script programs written by Guozhen Liu. Clones identified as having potential errors

**Table 1.** Recombinational Cloning Fidelity[a]

| | clone region analyzed | | |
|---|---|---|---|
| | 5RT1 | GSprimer | ORF |
| single base substitutions | 3 | 9 | 9 |
| deletion (1−7 bases) | 15 | 47 | 0 |
| total errors in region | 18 | 56 | 9 |
| total bases analyzed | 23 646 | 26 345 | 116 203 |
| error rate (errors/base) | $7.6 \times 10^{-4}$ | $2.1 \times 10^{-3}$ | $7.7 \times 10^{-5}$ |

[a] Numbers are based on sequence analysis of 1126 ORF-containing clones generated in the first round of cloning. 75 clones had errors in the indicated regions (illustrated in Figure 1). 5RT1, 5′ recombination tag; GSprimer, gene-specific primer region; ORF, position 100−200 of each ORF (less for smaller ORFs). Most (68) of the 75 error-containing clones contained one error.

were examined manually using the program Sequencher (GeneCodes, Ann Arbor, MI).

**Protein Expression and Western Analysis.** BL21 strains carrying the pTLJ03-derived *C. jejuni* expression clones were grown overnight at 37 °C in LB medium containing 100 $\mu$g/mL carbenicillin and 1% glucose. An aliquot (~80 $\mu$L) of the cultures was diluted in 1.2 mL of fresh medium resulting in an $OD_{600}$ of 0.2 and grown at 25 °C until the $OD_{600}$ reached approximately 0.9. IPTG was then added to a final concentration of 1 mM and the culture incubation continued at 25 °C for another 1.5 h. A 100-$\mu$L aliquot was mixed with 10 $\mu$L of Popculture reagent (Novagen, Madison, WI) containing lysozyme, nuclease, and protease inhibitors. Total protein samples were prepared in 2× Laemmli sample buffer and subjected to SDS-PAGE using 26-well 4−15% gradient gels (Bio-RAD, Hercules, CA). The proteins were transferred to PVDF membrane (Bio-RAD, Hercules, CA), blocked with 5% nonfat milk, probed with GST (Zymed, South San Francisco, CA) or 6× His (BD Biosciences, Palo Alto, CA) monoclonal antibody followed by anti-mouse secondary antibody (Bio-RAD, Hercules, CA) and the signal detected using the ECL-plus system (Amersham Biosciences Corp, Piscataway, NJ).

## Results and Discussion

To clone an ORF by in vivo recombination in *E. coli*, sequences must be added to each end of the ORF that are identical to the sequences flanking the cloning site in the vector. We refer to these homologous sequences between the insert and the vector as recombination tags. To make in vivo recombination cloning useful for high-throughput projects, the length of the recombination tags must be minimized to reduce the expense of generating primers for a large number of ORFs. A key problem with achieving this goal is that the efficiency of cloning, defined as the fraction of *E. coli* transformants that contain a vector with an insert, has been shown to decrease as the length of homology between the vector and insert is decreased.[6] In preliminary experiments we demonstrated that 19 and 20 bp recombination tags were sufficient to clone a variety of ORFs with good efficiency (67% or better) (Supporting Table 1). This was promising compared to previously published cloning efficiencies that used tags in this size range.[6,7] In addition, we observed similar cloning efficiencies when using three different *E. coli* strains (BUN10 (*recBC*, *sbcA*), KC8, and MG1655) (Supporting Table 1), although strain KC8 generally produced more recombinant colonies (data not shown). We concluded from these studies that relatively short recombination tags (approximately 20 bp) are sufficient for the efficient cloning of inserts in *E. coli*.

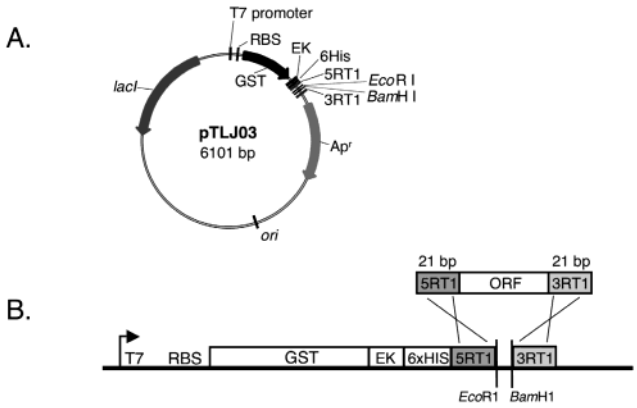


**Figure 1.** Recombinational cloning and expression vector pTLJ03. (A). Map of pTLJ03. 5RT1, 21 bp 5′ recombination tag; 3RT1, 21 bp 3′ recombination tag; RBS, ribosome binding site; EK, enterokinase cleavage site. (B). The expression and cloning regions of pTLJ03. PCR amplified ORFs flanked with 5RT1 and 3RT1 sequences, together with the cloning vector linearized at the *Eco*RI and *Bam*H1 sites can be co-transformed into chemically competent *E. coli*. Native intracellular recombination functions of *E. coli* will directionally insert the ORF into the vector. The resulting plasmid can be selected using the antibiotic resistance encoded on the plasmid.

To demonstrate that in vivo recombination in *E. coli* could be used for high-throughput cloning, we set out to clone the predicted ORFs of *C. jejuni* into an *E. coli* expression vector, pTLJ03 (Figure 1A). Such a clone set would enable expression and purification of GST-His-tagged *C. jejuni* proteins that could be used for a variety of functional studies. We designed gene-specific primer pairs to amplify the full length ORFs, from ATG to stop codon, for the 1685 genes predicted from the *C. jejuni* NCTC11168 genome sequence.[12] The 5′ and 3′ primers included 21 base recombination tags, 5RT1 and 3RT1, that are identical to the regions of pTLJ03 flanking the insertion site (Figure 1B). We used the primer pairs in 1685 PCR reactions with *C. jejuni* genomic DNA as template. As judged by DNA gel electrophoresis (Supporting Table 2 and Supporting Figure 1), we successfully amplified 1471 (87.3%) of the predicted ORFs.

We performed two rounds of recombination cloning. The first round included transformations for all of the PCR-amplified inserts, regardless of whether a product had been detected on an agarose gel. We used PCR to assess an ampicillin-resistant colony from each transformation for the presence of vector containing insert. For the transformations involving PCR products that had been visible on a gel, 75.3% (1013 out of 1346) of the colonies had the vector with an insert. Some of the PCR products that had not been visible on a gel were also successfully inserted (20 total). In cases where the first colony contained a vector without an insert, or the PCR validation reaction failed, we tested a second colony from the same transformation. This led to an additional 269 clones with inserts. Combined, these results indicated that we had cloned 1302 *C. jejuni* ORFs into pTLJ03.

We performed a second round of cloning for the ORFs that failed to clone in the first round. To generate additional insert DNA and to increase the length of identity between insert and vector, we amplified the primary PCR products using a primer pair that included the 5RT1 and 3RT1 sequences as well as an additional 23 bases of contiguous pTLJ03-specific sequences at either end. We obtained PCR products for 280 reactions as judged by gel electrophoresis (data not shown). Using these

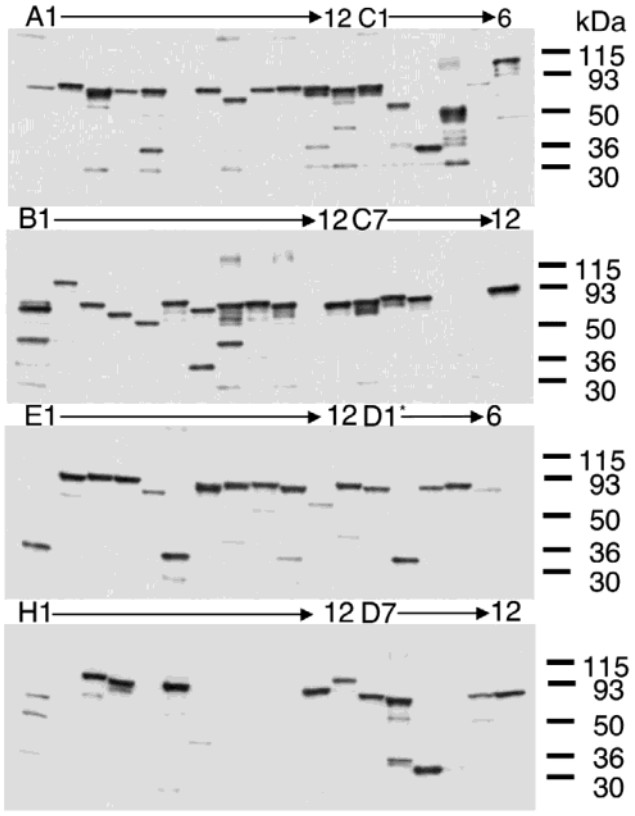


**Figure 2.** Analysis of *C. jejuni* clone expression. Western analysis using anti-GST antibodies. The sample corresponding to D2 was not included in this analysis. Predicted protein sizes for each position are listed in Supporting Table 4.

secondary PCR products, we successfully cloned 263 additional ORFs. The combined results from the two rounds of cloning indicated that we had cloned a total of 1565 (92.9%) *C. jejuni* ORFs. The successfully cloned ORFs ranged in size from 93 to 4020 bp and the cloning efficiency appeared to be independent of insert size (Supporting Figure 2).

To assess the fidelity of recombinational cloning, we analyzed the DNA sequences of the clones to detect possible errors. We focused our attention on the vector sequence immediately upstream of the 5′ recombination tag, the 5RT1 sequence itself, the ORF-specific region of the 5′ primer sequence, and a 100 bp internal section of each ORF. Of the 1126 clones that we analyzed from the first round of cloning, only 75 had a sequence that differed from the predicted sequence in one or more of these regions, indicating an overall cloning fidelity of 93.3% (Supporting Table 3). A similar rate of error-free clones was obtained in the second round of cloning (Supporting Table 3). The 75 clones with sequence errors had either a single base substitution or a small deletion. Examination of the distribution of these errors across the different regions of the clones indicated that the recombination event itself occurs with high fidelity (Table 1). No errors were detected in the region upstream of 5RT1, whereas most of the errors occurred in the primer regions. The fact that the highest error rates (errors/bp) were observed in both the 5RT1 and gene-specific regions of the primer sequence suggested that the errors occurred independently of the recombination event, since the gene-specific primer region does not share identity with the vector. Furthermore, the error rate observed for the ORF-encoding sequence (downstream of the gene-specific primer region), was significantly less than that observed for the primer regions. This implies that most of the errors in the primer regions were not due to DNA polymerase during PCR amplification of the ORFs, or any other reason that would apply to the entire ORF. Together, these analyses support the idea that the primary source of errors in this clone set was oligo synthesis rather than the recombinational cloning event.

To demonstrate that the ORFs cloned into pTLJ03 via recombinational cloning could be expressed as GST-His-tagged fusion proteins, we transformed a subset into *E. coli* strain BL21 (DE3) and performed immunoblotting. GST-6His fusion proteins were expressed as expected (Figure 2) (Supporting Figure 3). These results indicate that this *C. jejuni* expression clone set will be useful for a variety of applications that require purified recombinant proteins, including protein interaction, activity, and structural studies.

## Conclusions

The ability to easily construct new clone sets has become increasingly important, as multiple techniques are being developed and applied to obtain more thorough proteome characterizations. As illustrated in this study, large clone sets can be generated using nothing more than the native homologous recombination functions of *E. coli* to insert tagged ORFs into vectors. This simple method is efficient and requires no in vitro enzymatic steps. The fidelity of the process is excellent; errors are rare and appear to be introduced primarily during oligo synthesis and PCR amplification, steps in common with most high-throughput cloning procedures. There are no particular sequence requirements of the recombination tags; therefore, tags might be engineered for use with most any vector of interest. Also with this method there is the potential to clone one set of recombination-tagged ORFs into multiple vectors that carry the matching tag sequence at their cloning sites. Alternatively, this in vivo recombination approach can be used to quickly construct entry clones in preparation for subcloning into a variety of vectors using in vitro site-specific recombinase systems.[1,2] Taken together, these features make in vivo recombinational cloning in *E. coli* a useful approach for generating large clone sets for proteome characterization.

**Acknowledgment.** We thank Victor J. DiRita (University of Michigan-Ann Arbor) for providing genomic *C. jejuni* DNA, David DeWitt and the Michigan State University genomics core facility for sequencing, and Dima El-Khechen and Bernie Mangiola for expert technical assistance. We thank Dan Liu for writing the Perl scripts to facilitate primer design. We also thank Keith Gulyas and Kyle Gardenour for critical reading of the manuscript, and Phillip Andrews and Linda Mansfield for helpful discussions. This work was supported by the Michigan Life Sciences Corridor funding to the Michigan Proteome Consortium, and by the National Institutes of Health grant no. R01HG01536.

**Supporting Information Available:** Supporting Information including three figures, four tables, and a description of pTLJ02 is available free of charge via the Internet at http://pubs.acs.org.

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PR0341134