Benchmarks

A bacterial two-hybrid system that utilizes Gateway cloning for rapid screening of protein-protein interactions

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Comprehensive clone sets representing the entire genome now exist for a large number of organisms. The Gateway entry clone sets are a particularly useful means to study gene function, given the ease of introduction into any Gateway-suitable destination vector. We have adapted a bacterial two-hybrid system for use with Gateway entry clone sets, such that potential interactions between proteins encoded within these clone sets can be determined by new destination vectors. We show that utilizing the Gateway clone sets for *Francisella tularensis* and *Vibrio cholerae*, known interactions between *F. tularensis* IglA and IglB and *V. cholerae* VipA and VipB could be confirmed with these destination vectors. Moreover, the introduction of unique tags into each vector allowed for visualization of the expressed hybrid proteins via Western immunoblot. This Gateway-suitable bacterial two-hybrid system provides a new tool for rapid screening of protein-protein interactions.

Recently, complete sets of clones representing ORFs of a number of species have been constructed (1–3) utilizing Gateway entry clones and are available to investigators through the Pathogen Functional Genomics Resource Center (PFGRC; <http://pfgrc.jcvi.org>). Gateway cloning technology (Invitrogen, Carlsbad, CA, USA) uses λ recombination proteins to circumvent traditional restriction enzyme/ligation cloning (4). ORFs are introduced into Gateway “entry vectors” and can then be moved into any appropriate Gateway-compatible “destination vectors” via λ -mediated recombination. The recombination reaction is rapid and allows for directional cloning that maintains reading frame.

We have developed new Gateway destination vectors for use in a bacterial two-hybrid system developed to detect interactions between fusions to a DNA binding protein and an RNA polymerase (RNAP)–binding protein (5–7). In the modified version of this system (8), interaction between a protein fused to Zif (Zinc-finger DNA binding domain from the murine Zif268 protein) and a protein fused to the ω subunit of *Escherichia coli* RNAP facilitates transcriptional activation of a Zif-dependent *lacZ* reporter gene.

Type VI secretion systems (T6SSs) are recently described protein assemblies that facilitate secretion across Gram-negative bacterial membranes into the extracellular milieu and into eukaryotic cells (9–12).

The *V. cholerae* T6SS components VipA and VipB are essential for secretion and interact with each other to form a tubular complex (13,14). Homologs of VipA and VipB are found in most T6SS gene clusters, including IglA and IglB of *F. tularensis*, which are encoded within the *Francisella* pathogenicity island and are essential for virulence. IglA and IglB have been shown to interact via immunoprecipitation (15) and a yeast two-hybrid system (14). The IglA/VipA interaction was therefore an ideal means to validate the new Gateway vectors designed to identify protein-protein interactions.

Incorporation of an ORF from a Gateway donor plasmid into pKEK1286 (pKEK1286 and pKEK1287 are described in the Supplementary Materials) results in a protein fusion at its C terminus with the zinc-finger DNA binding protein Zif; it also incorporates a FLAG epitope at its N terminus (Figure 1). Incorporation of an ORF from a Gateway donor plasmid into pKEK1287 results in a protein fusion at its C terminus with the RNA polymerase ω subunit; it also incorporates a c-myc epitope at its N terminus.

We tested the utility of these bacterial two-hybrid destination vectors by examining protein-protein interactions between the *F. tularensis* IglA and IglB proteins. Entry clones containing IglA and IglB from the *F. tularensis* Gateway library at the PFGRC were introduced into pKEK1286 and pKEK1287, and the resulting protein fusions were analyzed for protein-protein interactions. Because the Gateway λ recombination reactions require surrounding sequences that result in the introduction of additional amino acids between the ORF and the epitope tag and fusion partner (Zif or ω) (Figure 1), we also introduced *iglA* and *iglB* into the original bacterial two-hybrid vectors by traditional cloning techniques (see Supplementary Materials) to determine if there were any negative effects of the linker regions in the Gateway-derived fusions. We then tested the cloned fusions (IglA^C- ω and IglB^C-Zif) along with the Gateway-derived IglA and IglB fusions (IglA^G-Zif, IglA^G- ω , IglB^G-Zif, and IglB^G- ω) for their ability to interact (Figure 2).

The IglA-Zif and IglB- ω protein fusions created by restriction/ligation (IglA^C-Zif, IglB^C- ω) interact and stimulate transcription from the engineered promoter-*lacZ* fusion present in the KDZif1ΔZ *E. coli* reporter strain, as anticipated. Likewise, the IglA-Zif + IglB- ω and IglB-Zif + IglA- ω protein fusions created in the new Gateway destination vectors (IglA^G-Zif + IglB^G- ω and IglA^G- ω + IglB^G-Zif) also interact and

Figure 1. Bacterial two-hybrid Gateway destination vectors. Shown are the attributes of pKEK1286 and pKEK1287 (top) and sequences of the expected products following the Gateway recombination reaction into these plasmids (bottom).

Figure 2. Gateway bacterial two-hybrid analysis of *F. tularensis* IgIA and IgIB interaction. *E. coli* reporter strain KDZif1ΔZ was transformed with empty vectors pACTR-AP-Zif and/or pBRGPω (-), and plasmids pKEK1249 (IgIB^C-Zif), pKEK1254 (IgIA^C-ω), pKEK1416 (IgIA^G-Zif), pKEK1415 (IgIB^G-ω), pKEK1417 (IgIA^G-ω), and/or pKEK1414 (IgIB^G-Zif), and assayed for β-galactosidase activity. C superscript denotes cloned protein fusions, while G superscript denotes protein fusions created by Gateway LR reaction. All values result from triplicate samples, and IgIA + IgIB interactions gave values significantly above background (either empty vectors or single plasmid paired with empty vectors; $P < 0.001$, Student's *t*-test).

stimulate transcription in the reporter strain; the interaction is independent of whether IgIA or IgIB is fused to Zif or ω. Either IgA-Zif or IgB-ω fusions paired with empty vectors resulted in background levels of activity.

The Gateway-derived fusions exhibited reduced activity when compared with the cloned fusions. Pairing a Gateway-derived protein fusion with its cognate cloned protein fusion (IgIA^C-Zif + IgIB^G-ω and IgIA^G-Zif + IgIB^C-ω; Figure 2) led to an intermediate level of activity. We can

conclude that this lower activity is linked to the Gateway-derived protein fusions and is due to the presence of the additional amino acids in these fusions introduced in the linker regions required for λ recombination and/or the epitope tags on the N termini (Figure 1). Still, the interaction between the Gateway-derived protein fusions is significantly elevated above background and therefore provides a useful new tool to study protein-protein interactions.

We further measured protein-protein interactions between the *V. cholerae* VipA

and VipB proteins, which are homologs of the *F. tularensis* IgIA and IgIB proteins (13). Entry clones containing VipA and VipB from the *V. cholerae* Gateway library were introduced into pKEK1286 and pKEK1287, and the resulting protein fusions were analyzed for protein-protein interactions (Supplementary Figure S1A). The Gateway-derived IgIA-ω and IgIB-Zif fusions were utilized as interacting controls. The VipA-ω and VipB-Zif fusion proteins interact and stimulate significant β-galactosidase activity. No cross-species

interactions could be detected between IglA- ω and VipB-Zif or between IglB-Zif and VipA- ω ; the level of β -galactosidase activity in the reporter strain containing these fusions was not significantly different than the activity in strains containing just VipA- ω or VipB-Zif. This confirms previous results, where no interactions could be detected between IglA and VipB or IglB and VipA (14).

The N-terminal epitope tags in the Gateway-derived protein fusions were introduced to facilitate detection via Western immunoblot. To determine the utility of these tags, whole cell lysates of mid-log cultures used to measure VipA-VipB interactions were subjected to Western immunoblot analysis with anti-FLAG and anti-cmyc monoclonal antibodies (Supplementary Figure S1B). Specific detection of the fusion proteins was observed with anti-c-myc (~33 kDa) and anti-FLAG (~72 kDa) antibodies, as anticipated.

The new bacterial two-hybrid Gateway destination vectors described here should prove useful in the screening of complete genome Gateway clone libraries for protein-protein interactions. The ease of incorporation of Gateway entry clone ORFs into destination vectors should allow high-throughput techniques to be utilized in combination with these vectors.

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Competing interests

The authors declare no competing interests.

References

1. Matsuyama, A. and M. Yoshida. 2009. Systematic cloning of an ORFeome using the Gateway system. *Methods Mol. Biol.* 577:11-24.
2. Aguiar, J.C., J. LaBaer, P.L. Blair, V.Y. Shamailova, M. Koundinya, J.A. Russell, F. Huang, W. Mar, et al. 2004. High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* 14:2076-2082.
3. Gao, H., D. Pattison, T. Yan, D.M. Klingeman, X. Wang, J. Petrosino, L. Hemphill, X. Wan, et al. 2008. Generation and validation of a *Shewanella oneidensis* MR-1 clone set for protein expression and phage display. *PLoS One* 3:e2983.
4. Walhout, A.J., G.F. Temple, M.A. Brasch, J.L. Hartley, M.A. Lorson, S. van den

- Heuvel, and M. Vidal. 2000. Gateway recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.* 328:575-592.
5. Dove, S.L. and A. Hochschild. 1998. Conversion of the omega subunit of *Escherichia coli* RNA polymerase into a transcriptional activator or an activation target. *Genes Dev.* 12:745-754.
6. Dove, S.L., J.K. Joung, and A. Hochschild. 1997. Activation of prokaryotic transcription through arbitrary protein-protein contacts. *Nature* 386:627-630.
7. Joung, J.K., E.I. Ramm, and C.O. Pabo. 2000. A bacterial two-hybrid selection system for studying protein-DNA and protein-protein interactions. *Proc. Natl. Acad. Sci. USA* 97:7382-7387.
8. Vallet-Gely, I., K.E. Donovan, R. Fang, J.K. Joung, and S.L. Dove. 2005. Repression of phase-variable cup gene expression by H-NS-like proteins in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 102:11082-11087.
9. Pukatzki, S., A.T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W.C. Nelson, J.F. Heidelberg, and J.J. Mekalanos. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. USA* 103:1528-1533.
10. Mougous, J.D., M.E. Cuff, S. Raunser, A. Shen, M. Zhou, C.A. Gifford, A.L. Goodman, G. Joachimiak, et al. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526-1530.
11. Ma, A.T., S. McAuley, S. Pukatzki, and J.J. Mekalanos. 2009. Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* 5:234-243.
12. Barker, J.R., A. Chong, T.D. Wehrly, J.-J. Yu, S.A. Rodriguez, J. Liu, J. Celli, B.P. Arulanandam, and K.E. Klose. 2009. The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. *Mol. Microbiol.* 74:1459-1470.
13. Bonemann, G., A. Pietrosiuk, A. Diemand, H. Zentgraf, and A. Mogk. 2009. Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *EMBO J.* 28:315-325.
14. Broms, J.E., M. Lavander, and A. Sjostedt. 2009. A conserved alpha-helix essential for a type VI secretion-like system of *Francisella tularensis*. *J. Bacteriol.* 191:2431-2446.
15. de Bruin, O.M., J.S. Ludu, and F.E. Nano. 2007. The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth. *BMC Microbiol.* 7:1.

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