ASD-GFP Vectors for In Vivo Expression Technology in *Pseudomonas aeruginosa* and Other Gram- Negative Bacteria

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ABSTRACT

We describe the construction of promoter probe vectors designed for identification of bacterial genes induced in vitro and/or in vivo and for measurement of gene expression levels for in vivo expression technology. These plasmids use the *Pseudomonas aeruginosa* aspartate β -semialdehyde dehydrogenase (*asd*) gene as a selectable marker and *b*-galactosidase (*pIVPRO*, 10.88 kb) or mutant green fluorescent protein with enhanced fluorescence properties (*mut3GFP*, *pIVET-GFP*, 5.48 kb) as reporter gene systems. The proposed strategies can be adapted for use in most Gram-negative bacteria.

INTRODUCTION

In vitro laboratory conditions do not perfectly mimic the environments encountered during an infection (18). In parallel, most virulence-associated genes are not constitutively expressed but rather coordinately regulated by environmental signals. Therefore, strategies have been designed for selection of genes uniquely expressed or induced in vivo, but not detectable by in vitro methods (2,12,16,17).

Aspartate β -semialdehyde dehydrogenase (ASD) is an essential enzyme in the biosynthesis of diaminopimelate (DAP), a component of the peptidoglycan in the cell wall of Gram-negative bacteria (23). The product of the *asd* gene is part of the biosynthetic pathway for lysine, methionine and threonine (27). In the absence of DAP, ASD-deficient bacteria undergo cell lysis. Because mammalian tissues contain no DAP, the *asd* gene could provide stringent selection in in vivo expression technology (IVET) (8,9). Vaccine

delivery systems based on stringent requirements for DAP have previously been used for in vivo complementation of ASD-attenuated bacteria (8,20,32).

The green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has been cloned and expressed in bacteria, and its three-dimensional structure has been determined at 1.9-Å resolution (21). The wild-type GFP of 238 amino acids absorbs blue light at 395 nm (with a minor peak at 470 nm) and emits green light at 509 nm maximum (a shoulder at 540 nm) (19,21,22,30). Mutant GFPs have enhanced fluorescence emission, higher solubility and improved chromophore formation kinetics (4,5). GFP has excellent characteristics for natural fluorescence in eukaryotes and in prokaryotes without exogenous substrates or cofactors and is stable with minimal photobleaching at 470 nm. In vivo fluorescence persists in formaldehyde-fixed specimens, and GFP is known as an excellent reporter (1,3,6,7,11,15,28,31).

IVET vectors were designed for identification and qualitative estimation of in vivo- and/or in vitro-induced genes. The ASD-GFP and ASD-LACZ phenotypes are alternatives to thymine and purine metabolism selection schemes found in previously described *pIVET1* and *pIVET2* vectors (16,17).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used for genetic constructs have been described previously (4,6,13,14,16,17, 25,26,29).

In Vitro DNA Manipulations

As shown in Figure 1, directional cloning of the 1.13-kb *XbaI/KpnI* DNA fragment encoding the promoterless *asd* gene from *pPS445* into the mobilizable suicide vector *pGP704* generated *pMON2200* (13,15). The 6.1-kb *SphI* DNA fragment containing the promoterless *lacZY* gene from *pIVET1* was subcloned into *pMON2200* in the same orientation as *asd* and generated *pIVPRO*. The 0.74-kb *KpnI/SphI* DNA fragment (*mut3gfp* gene) from *pKEM*

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(4,28) plasmid was cloned into pMON2200 previously digested with *Kpn*I/*Sph*I, creating pIVET-GFP.

Construction of Libraries

Pseudomonas aeruginosa PAO1 genomic DNA was partially digested with *Mbo*I, size-selected by agarose gel electrophoresis (1–4 kb) and cloned into the *Bgl*III site 5' to the promoterless *asd-lacZY* (pIVPRO) or *asd-mut3gfp* (pIVET-GFP). The random pool of fusions was initially maintained in *E. coli* DH5 α (λ pir) (14,16) and transformed in *E. coli* SM10(λ pir) (13) for bacterial conjugation to *P. aeruginosa* (Δ *asd*::Gm).

Selection of Recombinants

E. coli electroporants were selected on TSA plates (Difco, Detroit, MI, USA) containing 100 μ g/mL ampicillin. Plasmid pPS445 was maintained in *E. coli* SH309 (Δ *asd*) (24) and plasmid pKEM derivatives in *E. coli* DH5 α TM (Life Technologies, Gaithersburg, MD, USA). Plasmids pGP704, pMON2200, pIVET-GFP and pIVPRO were maintained in *E. coli* DH5 α (λ pir), and *E. coli* SM10(λ pir) was used for conjugation. *Pseudomonas* libraries were selected on TSA-containing carbenicillin (Cb; 500 μ g/mL), either with 500 μ g/mL DAP or without DAP. β -Galactosidase expression was estimated by plating libraries on TSA plates containing 40 μ g/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Colonies were examined under UV light for GFP expression.

RESULTS AND DISCUSSION

Design and Properties of pIVET-GFP and pIVPRO Vectors

Plasmids were constructed as described in Materials and Methods using the pGP704 suicide vector having the R6K origin of replication and requiring *pir*-encoded π protein, ampicillin resistance as a selectable marker and the RP4 *mob* for mobilization from Tra⁺ strains (14,25). The final constructs are depicted in Figure 1. The vectors constructed in this study differed from the reported IVET vectors of Mahan et al. (16,17) and Slauch et al. (26) in various

Table 1. Comparison of Identity Values (%) between the ASD Polypeptide Sequence of *Pseudomonas aeruginosa* with ASD from Other Bacterial Species^a

	<i>Escherichia coli</i>	<i>Haemophilus influenzae</i>	<i>Bordetella pertussis</i>	<i>Neisseria meningitidis</i> ^b
<i>P. aeruginosa</i> ^a	68.3	65.9	67.9	67.6
<i>E. coli</i>		72.5	66.7	65.5
<i>H. influenzae</i>			64.7	64.0
<i>B. pertussis</i>				66.4

^aGenBank® Accession Nos.: *E. coli*, V00262; *H. influenzae*, L45287 and L42023; *B. pertussis*, X75813; *N. meningitidis*, Z14063; *P. aeruginosa*, U11055.

^bPartial sequence.

Figure 1. Construction of promoter probe vectors pIVET-GFP and pIVPRO. The locations of the ampicillin-resistance (*Ap*^r) gene ("Ap"), plasmid R6K origin of replication ("oriR6K") and mobilization fragment ("mobRP4") constituting the suicide plasmid pGP704 are shown in open white boxes. The black arrow indicates the location and orientation of the promoterless *P. aeruginosa asd* gene. The gray arrow indicates the location and orientation of the promoterless *gfp* gene, and the dark-gray arrow indicates the promoterless *lacZY* genes.

aspects. Originally, pIVET1 and pIVET2 selection was based on in vivo complementation of *purA* and *thy*, respectively. The products of these genes are essential for purine and pyrimidine biosynthesis, and their absence severely attenuated growth in vivo. Although they proved useful and adequate for *Salmonella typhimurium* in a mouse model of septicemic infection (16,17, 26), there have been concerns about selection stringencies and potential leakiness in other bacterial systems. Not all types of infections would necessarily deprive bacteria of purines or pyrimidines. These nutrients could be scavenged by bacteria at sites of local inflammations or in tissues damaged by toxins. For plasmids pIVET-GFP and pIVPRO, the *P. aeruginosa asd* gene was chosen because: (i) ASD selection is stringent and non-leaky; (ii) DAP is absent from mammalian tissues, and bacterial *asd* mutants undergo rapid lysis; (iii) alignments of amino acid sequences of ASD from *P. aeruginosa* (13) with ASD polypeptide sequences from other Gram-negative bacteria confirm that ASD is conserved in evolution (Table 1); (iv) complete *asd* deletion mutants are available for *E. coli* (20,24), *S. typhimurium* (8,20), *Neisseria meningitidis* (9) and *P. aeruginosa* (13); (v) the GC-rich *P. aeruginosa asd* is a heterologous system, thus minimizing the possibility of homologous recombination between the IVET *asd* gene and *asd* genes from the bacterial chromosome of an *asd*-host strain.

The *lacZY* genes were chosen as IVET reporters for their sensitivity, reliability, ease of in vitro measurements and availability of Δlac host strains. However, the 6.1-kb size of *lacZY* in pIVPRO hampers construction of representative genomic libraries and subsequent manipulations of fusion plasmids. In pIVET-GFP, the much smaller 740-bp *mut3gfp* gene was therefore utilized. Expression of *mut3gfp* was visualized by inspecting colonies on agar plates using an inexpensive UV light or by spectrophotometry of small samples in suspension. One advantage of ASD-GFP IVET is the potential use of an automated fluorescence-activated cell-sorting apparatus for measurements of GFP expression observed in vivo (4). Mutant GFP is detectable within 8 min

after induction, compared to two hours for the wild-type enzyme, permitting studies of transient gene expression at early time points (4).

Testing of Novel IVET Vectors

The IVET system was tested by randomly cloning promoter-containing chromosomal *P. aeruginosa* PAO1 DNA fragments into the *Bgl*III site 5' to the promoterless *asd-lacZY* (pIVPRO-1) or *asd-mut3gfp* (pIVPRO-2) genes. The random pool of fusions was transferred and integrated into a Δasd deletion strain of *P. aeruginosa* ($\Delta asd::Gm$). The merodiploids were grown and selected on rich media containing carbenicillin, with or without DAP. The *LacZY* or GFP expression was assayed by plating libraries on TSA plates containing X-gal or by direct observation of the colonies under UV light irradiation, respectively. Growth on the DAP-containing medium of the random library resulted in mixed populations of Lac^+/Lac^- or GFP^+/GFP^- phenotypes. Growth on DAP-deprived medium of the same libraries resulted in the stringent selection of Lac^+ or GFP^+ colonies, demonstrating that expression of the promoterless *asd* gene could alleviate the DAP requirement of a Δasd mutant by selecting for constitutively expressed promoters. The pool of fusions has also been challenged in various animal models. Fusion strains transcriptionally active in vivo were also obtained, and details will be reported elsewhere.

The constructs described here are IVET-based plasmid vectors for selection of genes differentially expressed or expressed solely in vivo. Some bacterial pathogens show tissue tropism and/or follow precise steps during infection. Using the pIVET-GFP and pIVPRO vectors, one should be able to detect patterns in gene expression or to determine the genetic response to environmental stimuli. Furthermore, the pIVET-GFP vector can be used in vivo and the expression monitored in real time without invasive procedures based on the GFP properties. pIVET-GFP derivatives expressing variants of GFP with altered absorption spectra and re-emission levels will permit multiple IVET labelings. It is anticipated that

the ASD-LACZ and ASD-GFP vectors will expand and/or complement the repertoire of *ivi* genes obtained with the previously described IVET vectors (5,10,11).

REFERENCES

1. Arigoni, F., K. Pogliano, C.D. Webb, P. Stragier and R. Losick. 1995. Localization of protein implicated in establishment of cell type to sites of asymmetric division. *Science* 270:637-640.
2. Camilli, A. and J.J. Mekalanos. 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol. Microbiol.* 18:671-683.
3. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-804.
4. Cormack, B.P., R.H. Valdivia and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33-38.
5. Delagrange, S., R.E. Hawtin, C.M. Silva, M.M. Yang and D.C. Youvan. 1995. Red-shifted excitation mutants of the green fluorescent protein. *Bio/Technology* 13:151-154.
6. Dhandayuthapani, S., L.E. Via, C.A. Thomas, P.M. Horowitz, D. Deretic and V. Deretic. 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. *Mol. Microbiol.* 17:901-912.
7. Fey, P., K. Compton and E.C. Cox. 1995. Green fluorescent protein production in the cellular slime molds *Polysphondylium pallidum* and *Dictyostelium discoideum*. *Gene* 165:127-130.
8. Galán, J.E., K. Nakayama and R. Curtiss III. 1990. Cloning and characterisation of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. *Gene* 94:29-35.
9. Hatten, L.-A., H.P. Schweizer, N. Averill, L. Wang and A.B. Schryvers. 1993. Cloning and characterisation of the *Neisseria meningitidis asd* gene. *Gene* 129:123-128.
10. Heim, R., A.B. Cubitt and R.Y. Tsien. 1995. Improved green fluorescence. *Nature* 373:663-664.
11. Heim, R., D.C. Prasher and R.Y. Tsien. 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 91:12501-12504.
12. Hensel, M., J.E. Shea, C. Gleeson, M.D. Jones, E. Dalton and D.W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* 269:400-403.
13. Hoang, T.T., S. Williams, H.P. Schweizer and J. Lam. 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate- β -semialdehyde dehydrogenase. *Microbiology* 143:899-907.
14. Kolter, R., M. Inuzuka and D.R. Helinski. 1978. Trans-complementation-dependent re-

Short Technical Reports

- plication of a low molecular weight origin fragment from plasmid R6K. *Cell* 15:1199-1208.
15. **Kremer, L., A. Baulard, J. Estaquier, O. Poulain-Godefroy and C. Locht.** 1995. Green fluorescent protein as a new expression marker in *Mycobacteria*. *Mol. Microbiol.* 17:913-922.
16. **Mahan, M.J., J.M. Slauch and J.J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* 259:686-688.
17. **Mahan, M.J., J.W. Tobias, J.M. Slauch, P.C. Hanna, R.J. Collier and J.J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. *Proc. Natl. Acad. Sci. USA* 92:669-673.
18. **Mekalanos, J.J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* 174:1-7.
19. **Morin, J.G. and J.W. Hastings.** 1971. Energy transfer in a bioluminescent system. *J. Cell. Physiol.* 77:313-318.
20. **Nakayama, K., S.M. Kelly and R. Curtiss III.** 1988. Construction of an *asd+* expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Bio/Technology* 6:693-697.
21. **Ormö, M., A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien and S.J. Remington.** 1996. Crystal structure of the *Aequorea victoria* green fluorescence protein. *Science* 273:1392-1395.
22. **Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast and M.J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:229-233.
23. **Schleifer, K.H. and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* 36:407-477.
24. **Schweizer, H.P., G. Sweet and T.J. Larson.** 1986. Physical and genetic structure of the *glpD-malT* interval of the *Escherichia coli* K-12 chromosome. *Mol. Gen. Genet.* 202:488-492.
25. **Simon, R., U. Priefer and A. Puhler.** 1983. A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* 1:784-791.
26. **Slauch, J.M., M.J. Mahan and J.J. Mekalanos.** 1994. *In vivo* expression technology for selection of bacterial genes specifically induced in host tissues. *Methods Enzymol.* 235:481-492.
27. **Théze, J., D. Margarita, G.N. Cohen, F. Borne and J.C. Patte.** 1974. Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K-12. *J. Bacteriol.* 117:133-143.
28. **Valdivia, R.H. and S. Falkow.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol. Microbiol.* 22:367-378.
29. **Vieira, J. and J. Messing.** 1982. The pUC plasmid, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259-268.
30. **Ward, W.W., C.W. Cody, R.C. Hart and M. Cormier.** 1980. Spectrophotometric identity of the energy transfer chromophores in *Renilla* and *Aequorea* green-fluorescent proteins. *Photochem. Photobiol.* 31:611-615.
31. **Webb, C.D., A. Decatur, A. Teleman and R. Losick.** 1995. Use of green fluorescent protein for visualisation of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. *J. Bacteriol.* 177:5906-5911.
32. **Wu, S.G., D.W. Pascual, J.L. Vancott, J.R. McGhee, D.R. Maneval, M.M. Levine and D.M. Hone.** 1995. Immune responses to novel *Escherichia coli* and *Salmonella typhimurium* vectors that express colonisation factor antigen-I (CFA/I) of enterotoxigenic *Escherichia coli* in the absence of the CFA-I positive regulator CFAR. *Infect. Immun.* 63:4933-4838.

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²M. Handfield, H.P. Schweizer¹, M.J. Mahan², F. Sanschagrin, T. Hoang¹ and R.C. Levesque
Université Laval
Ste.-Foy, QC, Canada
¹*Colorado State University*
Fort Collins, CO
University of California
Santa Barbara, CA, USA