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 β-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-A 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 lacZ 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
(4,28) plasmid was cloned into pMON2200 previously digested with KpnI/SphI, creating pIVET-GFP.

Construction of Libraries

Pseudomonas aeruginosa PAO1 genomic DNA was partially digested with MboI, size-selected by agarose gel electrophoresis (1–4 kb) and cloned into the BglII site 5' to the promoterless asd-lacZY (pIVPRO) or asd-mut3gfp (pIVET-GFP). The random pool of fusions was initially maintained in E. coli DH5α(λpir) (14,16) and transformed in E. coli SM10(λpir) (13) for bacterial conjugation to P. aeruginosa (Δasd::Gm).

Selection of Recombinants

E. coli electroporants were selected on TSA plates (Difco, Detroit, MI, USA) containing 100 µg/mL ampicillin. Plasmid pPS445 was maintained in E. coli SH309 (Δasd) (24) and plasmid pKEM derivatives in E. coli DH5α(λpir) (Life Technologies, Gaithersburg, MD, USA). Plasmids pGP704, pMON2200, pIVET-GFP and pIVPRO were maintained in E. coli DH5α(λpir), and E. coli SM10(λpir) was used for conjugation. Pseudomonas libraries were selected on TSA-containing carbenicillin (Cb; 500 µg/mL), either with 500 µg/mL DAP or without DAP. β-Galactosidase expression was estimated by plating libraries on TSA plates containing 40 µg/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>
<thead>
<tr>
<th>Escherichia coli</th>
<th>Haemophilus influenzae</th>
<th>Bordetella pertussis</th>
<th>Neisseria meningitidis</th>
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⁹GenBank® Accession Nos.: E. coli, V00262; H. influenzae, L45287 and L42023; B. pertussis, X75813; N. meningitidis, Z14063; P. aeruginosa, U11055.

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

Figure 1. Construction of promoter probe vectors pIVET-GFP and pIVPRO. The locations of the ampicillin-resistance (Ap') 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 *thi*, 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 inflammation 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 *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 *lacZ*Y 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 *lacZ*Y 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 *BgIII* site 5’ to the promoterless asd-lacZ (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* (Δasd; 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 stages 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-LAC2 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**

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