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In Vivo-Induced Genes in *Pseudomonas aeruginosa*

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In vivo expression technology was used for testing *Pseudomonas aeruginosa* in the rat lung model of chronic infection and in a mouse model of systemic infection. Three of the eight *ivi* proteins found showed sequence identity to known virulence factors involved in iron acquisition via an open reading frame (called *pvdI*) implicated in pyoverdine biosynthesis, membrane biogenesis (FtsY), and adhesion (Hag2).

Pseudomonas aeruginosa is an opportunistic pathogen important in cystic fibrosis patients, for whom chronic *P. aeruginosa* infections remain the major cause of acute pneumonia, leading to debilitating lung malfunction and premature death. Although several *P. aeruginosa* virulence factors have been extensively studied in vitro, less is known about virulence factors during infection. Several approaches have been reported to allow the recovery, identification, and characterization of genes that are expressed in the host (2–4). We have utilized the in vivo expression technology (IVET) *purA* promoter trap system (5) to identify *P. aeruginosa* genes that are specifically induced during mucosal and/or systemic infections. Here, we present evidence that the DNA fragments cloned in the promoter trap carry *ivi* genes in both animal models used.

Generation of chromosomal cointegrated *P. aeruginosa* PAO909 library. A library of random genomic DNA fragments from *P. aeruginosa* were cloned to the promoterless *purA-lacZY* into pIVET1. Genomic DNA fragments from *P. aeruginosa* strain PAO1 from 1 to 4 kb were size selected, purified, ligated with pIVET1, and electroporated into *Escherichia coli* DH5αλpir (strains and plasmids are listed in Table 1). Analysis of 48 recombinant plasmids confirmed that 99% had different *P. aeruginosa* DNA fragments ranging between 1 and 4 kb (data not shown). This random pool of plasmids was transformed into *E. coli* SM10λpir and transferred by conjugation into the *purA* mutant *P. aeruginosa* strain PAO909. The resulting chromosomal cointegrated library was represented by at least 2×10^5 colonies of *P. aeruginosa* transformants.

Selection of *P. aeruginosa* in vivo-induced genes. The cointegrated PAO909 library was used to infect BALB/c mice weighing 18 to 20 g (a septicemia model) intraperitoneally with 10^6 to 10^7 bacteria/mouse and to infect Sprague-Dawley rats intratracheally with 10^5 bacteria enmeshed into agar beads per lung (a chronic lung infection model [1]). After incubation, bacteria recovered from mouse livers and rat lungs were plated on rich selective medium containing the sensitive chromogenic substrate, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). A collection of 100 *ivi* fusions were recovered from infected mouse livers and infected rat lungs (Fig. 1).

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Characterization of *ivi* genes. Plasmid preparations from in vivo-selected PAO909 clones were electroporated into *E. coli* DH5αλpir to allow plasmid rescue. Next, rescued plasmids which had unique restriction patterns and which gave a Lac$^-$ phenotype in vitro were selected for further analysis by DNA sequencing. *ivi* junctions were sequenced using primers homologous to the 5′ region of the *purA* gene. Similarity searches with the *P. aeruginosa* genome were performed at the National Center for Biotechnology Information using the uncompleted *P. aeruginosa* sequence genome database (http://www.pseudomonas.com). Bioinformatics analysis was done using GeneMark and software in the University of Wisconsin Genetics Computer Group package (version 10.0). We identified three *ivi* genes with homology to known sequences: *pvdD*, *ftsY*, and *hag2*. The remaining six *ivi* genes were open reading frames (ORFs) found to have no DNA or protein similarity (Table 2).

Strain 131-17, identified by IVET (henceforth IVET 131-17), contains an unidentified ORF of 15,450 nucleotides (named *pvdI*) coding for a 5,150-amino-acid synthetase having 43% identity with PvdD of *P. aeruginosa*. We refer to this synthetase gene located upstream and in the same orientation as *pvdD*. The PvdD pyoverdine synthetase is involved in the synthesis of the fluorescent siderophore pyoverdine that is essential for iron uptake (6, 7). The independent isolation of IVET 131-17 from both animal models reflects the relative importance of iron acquisition in the establishment and/or maintenance of *P. aeruginosa* mucosal and systemic infections. Large-scale isolation of candidate virulence genes of *P. aeruginosa* strain PAK identified the pyochelin receptor (*fptA*), known to be inducible under iron-deprived conditions, providing further evidence that animal host tissues are deficient in free iron due to the presence of high-affinity iron binding proteins like transferrin (12, 13).

IVET 134-21 carries an ORF that encodes a protein sharing 65% identity with *E. coli* FtsY, a docking protein that interacts with the prokaryotic signal recognition particle-like complex involved in protein targeting and membrane biogenesis (10). Several *ivi* genes are involved in bacterial membrane modifications, presumably in response to overcoming environmental stresses imposed on the pathogen during infection (4).

IVET 131-19 carries a predicted peptide which has 43% identity with hemagglutinin Hag2 of *Eikenella corrodens*, an oral bacterium found in dental plaque (9). Similarly, the adherence of *P. aeruginosa* to the mucosa of the oropharynx is believed to be the initial step in colonization of the lower respiratory tract (14). The *ivi* of IVET 131-19 in both infection

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5αλpir	F^- φ80 Δ*lacZ* ΔM15 *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ^- *gyrA96 relA1* Δ(*lacZYA-argF*)*U169*; recipient	J. J. Mekalanos, Harvard University
SM10λpir	F^- *araD* Δ(*lac pro*) *argE*(Am) *recA56* Rif^r *nalA*; recipient	J. J. Mekalanos, Harvard University
P. aeruginosa		
PAO1	Wild-type *P. aeruginosa*	B. W. Holloway, Monash University
PAO909	*purA pur-67 E79 tv-2*; transduction mutant of PAO910	Pseudomonas Genetic Stock Center
100	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$]; Lac^- control strain	This study
101	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$]; Lac^- control strain	This study
102	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$]; Lac^+ control strain	This study
131-8	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI131-8]	This study
131-14	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-131-14]	This study
131-15	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-131-15]	This study
131-17	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-131-17]	This study
131-19	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-131-19]	This study
134-21	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-134-21]	This study
152-1	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-152-1]	This study
153-1	PAO909 [$purA^+$ $lacZ^+Y^+(Am^+)$ pIVI-153-1]	This study
Plasmids		
pIVET1	*'purA-lacZY*; suicide plasmid pGP704 *oriR6K Mob bla pir*	5
pIVI-131-8	DH5αλpir rescued from 131-8; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-131-14	DH5αλpir rescued from 131-14; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-131-15	DH5αλpir rescued from 131-15; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-131-17	DH5αλpir rescued from 131-17; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-131-19	DH5αλpir rescued from 131-19; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-134-21	DH5αλpir rescued from 134-21; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-152-1	DH5αλpir rescued from 152-1; PAO1 DNA-*purA-lacZY* fusion	This study
pIVI-153-1	DH5αλpir rescued from 153-1; PAO1 DNA-*purA-lacZY* fusion	This study

models suggests a mucosal and systemic requirement for *P. aeruginosa* adhesins, as is the case for other mucosal and systemic infection models (4). Cross talk of virulence factors between different in vivo pathogenesis models has been described previously using plants as hosts to identify *P. aeruginosa* virulence factors (8). The remaining six *ivi* genes code for proteins having no significant homology to reported proteins found in databases.

Induction of fusions is required for in vivo survival. All eight *ivi* clones showed no or weak β-galactosidase activity when in vitro promoter activity was tested as described by Slauch et al. (11) (data not shown). Results shown in Fig. 1 indicate that the mutant *P. aeruginosa purA* strain PAO909 could not be recovered from mouse liver and rat lung tissues, confirming the efficacy of the selection in both animal models. Moreover, the eight *ivi* fusions showed a 10^3- to 10^5-fold growth advantage in both infection models. Thus, induction of all eight *ivi* fusions is required for survival in both animal models under conditions of the IVET selection.

These eight *ivi* genes were shown to be required for survival under the conditions of IVET selection in both animal models, suggesting that at least some host signals present during mouse systemic infection are also present in the rat respiratory mucosa. The propensity to isolate *ivi* genes coding for proteins related to the expression of surface proteins such as FtsY, PvdI, and Hag2 may suggest that they play a role in virulence by some unknown mechanisms. IVET selects bacterial *ivi* genes that presumably contribute to the in vivo fitness of the pathogen host tissues. Many of the *ivi* genes that have been recovered from several pathogens infecting a wide variety of animal models are unknown (4). The high possibility of recovering *ivi* genes of unknown function may reflect our limited knowledge of the bacterial functions required to survive during infection. Many of these presumably reflect the unique lifestyle of each individual pathogen during growth in the host and may not be shared by other pathogens. Thus, further studies on both known and unknown *P. aeruginosa ivi* gene products will contribute to a better understanding of the pathobiology of *P. aeruginosa* as an opportunistic pathogen.

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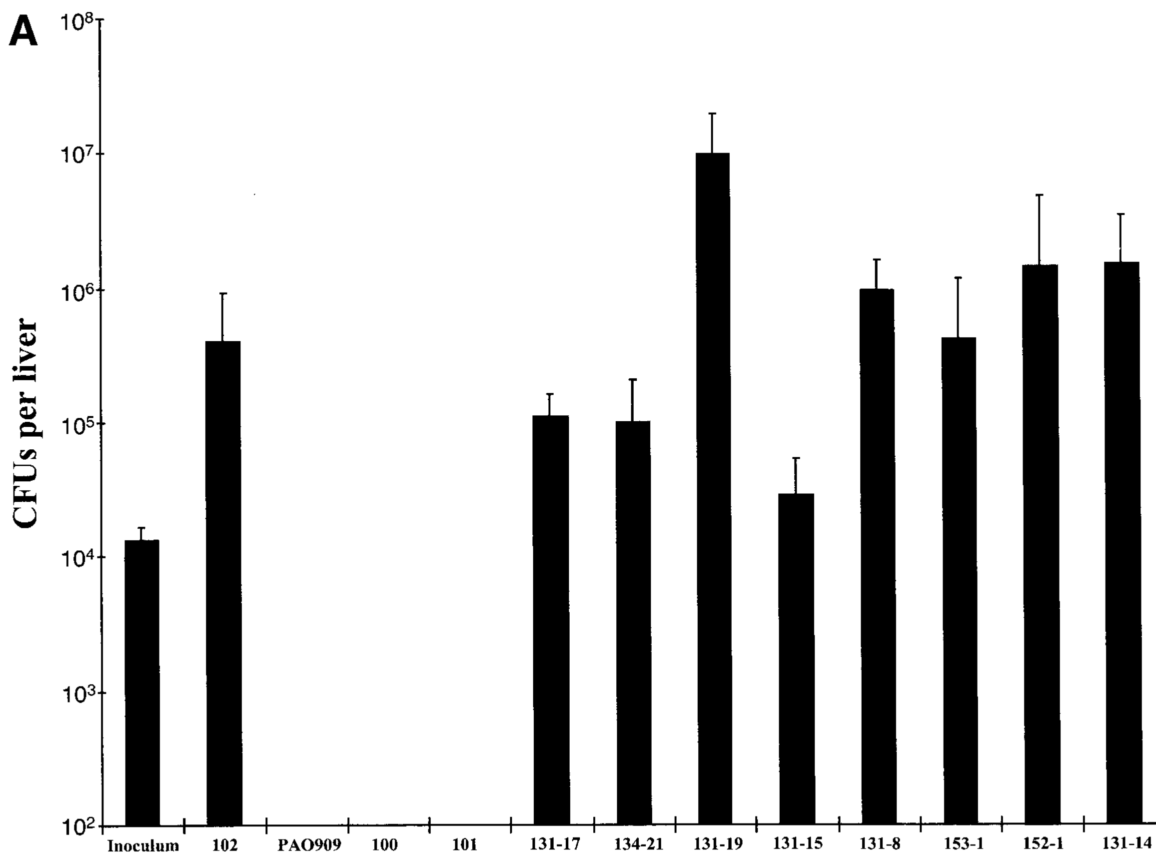
FIG. 1. Induction of *ivi* genes is required for survival in the animal. The vertical axis represents the number of CFU recovered from the organ of interest after inoculation. The inoculum bar represents the number of CFU of *P. aeruginosa* injected into each animal. Results are from the BALB/c mouse model of septicemia induced by intraperitoneal injection (10^4 CFU/mouse; 3 days) (A) and the Sprague-Dawley rat model of chronic lung infection induced via intratracheal instillation of bacterial cells enmeshed in agar beads (5×10^5 CFU/rat; 5 days) (B). Cells were grown overnight at 37°C in rich (adenine-supplemented) laboratory medium. Strains 100 and 101 (Lac^-) and strain 102 (Lac^+) were preselected *purA-lac* fusion strains. PAO909 is a *P. aeruginosa* auxotroph for adenine. Data are presented as averages of two to five independent assays ± standard deviations.


A
CFUs per liver
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Inoculum
102
PAO909
100
101
131-17
134-21
131-19
131-15
131-8
153-1
152-1
131-14

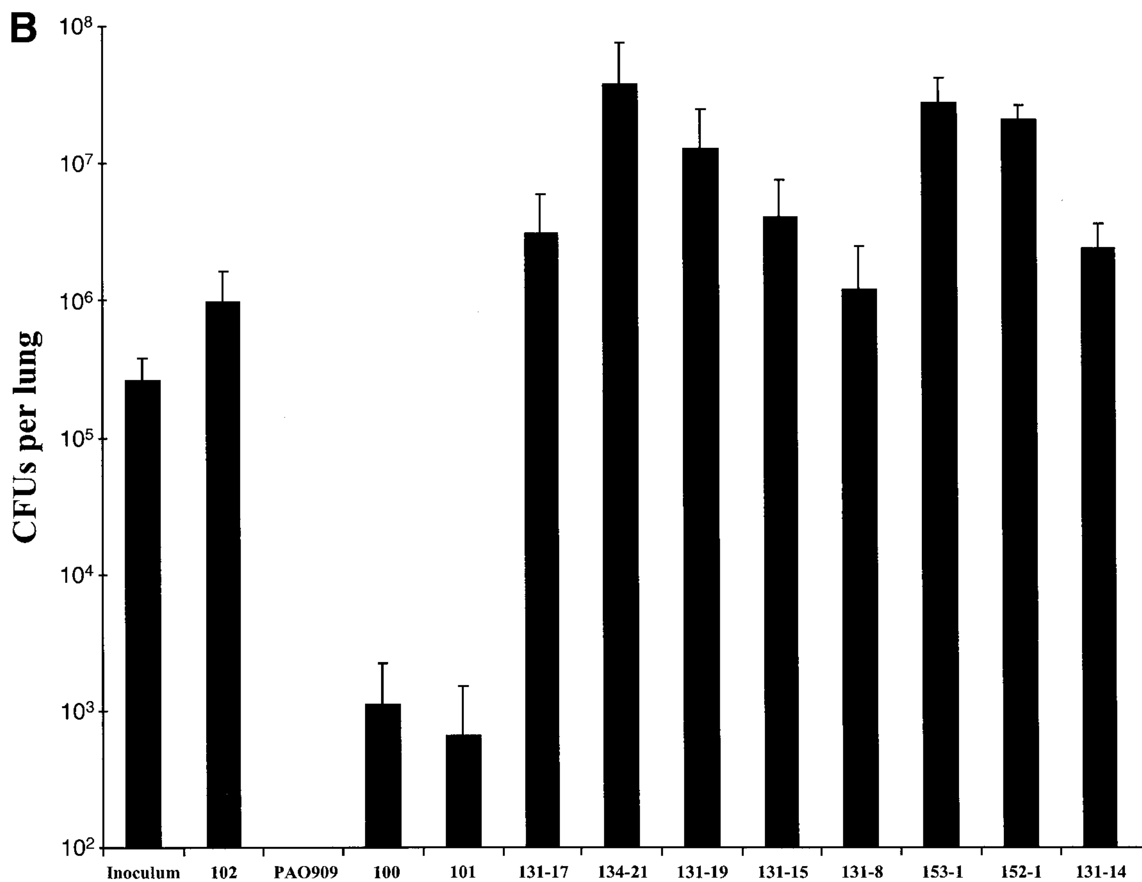

B
CFUs per lung
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Inoculum
102
PAO909
100
101
131-17
134-21
131-19
131-15
131-8
153-1
152-1
131-14

TABLE 2. *P. aeruginosa* in vivo-induced genes isolated in both animal models[a]

ivi fusion strain	Homolog	% Identity	Possible function	Possible role
Recovered from both animal models				
131-17	*P. aeruginosa* (PudD)	60	Pyoverdine biosynthesis	Iron scavenging
134-21	*H. influenzae* (FtsY)	66	Docking protein	Transport-secretion
131-19	*E. corrodens* (Hag2)	43	Adhesion	Colonization
131-15	ORF		Unknown	Unknown
153-1	ORF		Unknown	Unknown
131-8	ORF		Unknown	Unknown
Recovered exclusively from mouse model				
152-1	ORF		Unknown	Unknown
131-14	ORF		Unknown	Unknown

[a] GenBank accession numbers for these nucleotide sequences are AF214673 to AF214679.

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The first two authors (M.H. and D.E.L.) contributed equally to this work and are listed alphabetically.

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