Genetic footprinting with *mariner*-based transposition in *Pseudomonas aeruginosa*

Sandy M. Wong*† and John J. Mekalanos*‡§

*Department of Microbiology and Molecular Genetics, and ‡Shipley Institute of Medicine, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115

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The complete DNA sequence of *Pseudomonas aeruginosa* provides an opportunity to apply functional genomics to a major human pathogen. A comparative genomics approach combined with genetic footprinting was used as a strategy to identify genes required for viability in *P. aeruginosa*. Use of a highly efficient *in vivo* mariner transposition system in *P. aeruginosa* facilitated the analysis of candidate genes of this class. We have developed a rapid and efficient allelic exchange system by using the I-*Sce*I homing endonuclease in conjunction with *in vitro* mariner mutagenesis to generate mutants within targeted regions of the *P. aeruginosa* chromosome for genetic footprinting analyses. This technique for generating transposon insertion mutants should be widely applicable to other organisms that are not naturally transformable or may lack well developed *in vivo* transposition systems. We tested this system with three genes in *P. aeruginosa* that have putative essential homologs in *Haemophilus influenzae*. We show that one of three *H. influenzae* essential gene homologs is needed for growth in *P. aeruginosa*, validating the practicality of this comparative genomics strategy to identify essential genes in *P. aeruginosa*.

transposon | SCE jumping

The human pathogen *Pseudomonas aeruginosa* is the major cause of opportunistic infections in immunocompromised individuals and the primary cause of chronic pulmonary infections in patients with cystic fibrosis leading to respiratory failure and death. This organism is highly resistant to a broad range of antibiotics complicating clinical treatment (1–3). Identification of *P. aeruginosa* genes may provide us with an important set of candidates for potential targets of antimicrobial drugs. An approach has been developed recently to allow systematic identification of genes essential or conditionally essential for survival in *Haemophilus influenzae*, a human respiratory pathogen whose genome has been sequenced completely. The approach, termed GAMBIT (*G*enomic *A*nalysis and *M*apping *B*y *In vitro T*ransposition), exploits the use of the *mariner*-family transposon *Himar1* to produce transposon insertion mutants by *in vitro* transposition for subsequent functional genomic analyses (4). In this report, we demonstrate a strategy for essential gene identification in *P. aeruginosa* that makes use of *H. influenzae* functional genomics information.

Materials and Methods

Bacterial Strains, Plasmids, and Media. *Escherichia coli* strains SM10 λ pir, DH5α, and S17-1 (5) and *P. aeruginosa* strains were grown in LB broth and maintained by standard methods. *P. aeruginosa* strains created in this study were all derived from PAO1SR, a spontaneous streptomycin (Sm)-resistant isolate derived from the standard laboratory strain PAO1 (6). Sm provides an additional marker for selection. Antibiotics added to LB medium were as follows (in μg/ml): for *E. coli*, ampicillin, 100; kanamycin (Km), 50; gentamicin (Gm), 5; and chloramphenicol (Cm), 25; and for *P. aeruginosa*, carbenicillin (Cb), 300; Km, 500; Gm, 100; Cm, 200; and Sm, 200. Uracil (Ura) was used in LB or M9 minimal (Difco) plates at 100 μg/ml; 5-fluoroorotic acid (FOA) was used in LB plates at 300 μg/ml. Sucrose-resistant (Suc^r) *P. aeruginosa* isolates were screened on LB plates containing 5% (vol/vol) Suc. Standard molecular biology procedures were used for cloning and propagation of plasmids in *E. coli* (7). Plasmids were transferred conjugally from *E. coli* to *P. aeruginosa* on membrane filters with early-log-phase *E. coli* donors (≈10^8–10^9) and *P. aeruginosa* recipients (≈10^8) grown overnight at 42°C. Filters were incubated at 37°C on nonselective LB agar for a minimum of 5 h to overnight before plating mating mixture onto selective media.

Transposon, Plasmid, and Strain Construction. To create pFAC, the Km marker from pFD1 (8) was replaced with a 808-bp *Mlu*I fragment containing the *aacC1* Gm allele from pBSL182 (9). pSW(I-*Sce*I) was constructed by cloning a 714-bp *Sal*I (blunt ended)–*Nde*I fragment containing I-*Sce*I ORF from pCMV(I-*Sce*I+) (10) into the *Sma*I–*Nde*I sites of pJB658 (11). pSW(I-*Sce*I) and pJB658 (both carry ampicillin markers) were mobilized from *E. coli* S17-1 to PAO1SR to create strains SW658 and SWSce, respectively. Primers were designed with sequence information from the *Pseudomonas* Genome Project (http://www.pseudomonas.com). pSWΔFGm was constructed as follows: a 1-kilobase (kb) product was amplified from PAO1SR with primers MERF1 (5′-CGCGGATCCGCCATCCCGAATAGAGAGAAG) and 5′-CGTCACGCGTGAAATCCAGGGCGACGATGATGGG; a 1.12-kb product was amplified with primers 5′-ACGCGTGACGCCGGCTCCGATTACCT and MERF4 (5′-CGCGGATCCTTCCGGAATCACATAGTCGCGT) (*Bam*HI and *Mlu*I sites are underlined). The 1-kb and 1.12-kb products were used in PCR with primers MERF1 and MERF4 to amplify a 2.18-kb product, which was digested with *Bam*HI, Klenow end-filled, and cloned into the *Sma*I site of pEX100 (12) to create pSWΔF. The *aacC1* Gm cassette was cloned into the *Mlu*I site of pSWΔF, followed by removal of the *bla* gene by digesting with *Sca*I–*Ssp*I and Klenow end-filling to create pSWΔFGm. pSWkan was made by replacing a 324-bp *Sca*I–*Ssp*I fragment from the *bla* gene in pEX100 with a 840-bp *Sma*I fragment containing a Km marker from pUC18K (13). To create pSW1654–55, a 2.9-kb product was amplified from PAO1SR with primers 5′-CGCGGATCCTGGCAAGGCCCTGTCGCCGTAGA and MERPA1654 (5′-CGCGGATCCACAGCGACTGTCCAATCGACTCTC). This fragment was digested with *Bam*HI, Klenow end-filled, and cloned into the *Sma*I site of pSWkan. To create

Abbreviations: Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Cm, chloramphenicol; Cb, carbenicillin; FOA, 5-fluoroorotic acid; Ura, uracil; Suc, sucrose; ^{r}, resistant; ^{s}, sensitive; kb, kilobase.

†Present address: Department of Microbiology and Immunology, University of Michigan Medical School, 5641 Medical Science Building II, Ann Arbor, MI 48109.

§To whom reprint requests should be addressed. E-mail: jmekalanos@hms.harvard.edu.

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pSWΔ1655Gm (Δ807-bp from PA1655 ORF), pSW1654–55 was used in PCR to amplify an ≈8-kb product with primers 5′-CGACGCGTTGTCGTCGAGATTGCCGATCGGGGTCG and 5′-CGACGCGTTGCCGAGCTGCCGTTGAAGAAAGCC, followed by digesting with *Mlu*I and cloning of the *aacC1* Gm cassette into the *Mlu*I site. pSWΔ1654Gm (Δ688-bp from PA1654 ORF) was created as follows: primers 5′-TAGGGATAACAGGGTAATGGATCCAAGCTTTAGGGATAACAGGGTAAT and 5′-ATTACCCTGTTATCCCTAAAGCTTGGATCCATTACCCTGTTATCCCTA were annealed and cloned into the *Sma*I site of pSWkan to create pSWkanBH. Primers 5′-CGCGGATCCGCGGTACGGCTCATTCTTCAC and 5′-CGCGGATCCCGCGTTTCTTCTCGCAAAGAAG were used to amplify a 4.7-kb product from PAO1SR, which was digested with *Bam*HI and cloned into the *Bam*HI site of pSWkanBH to create pSW4.7. Primers 5′-CGACGCGTTGCCGAGCTGCCGTTGAAGAAAGCC and 5′-CGACGCGTTGTCGTCGAGATTGCCGATCGGGGTCG were used to amplify an ≈10-kb product from pSW4.7, which was digested with *Mlu*I followed by cloning of the *aacC1* Gm cassette into the *Mlu*I site. To create pSW906, a 4.1-kb PCR product was amplified from PAO1SR with primers MER906B2 (5′-CGCGGATCCTCGTGGTGTTCCAGCCAGTGAAATC) and MER906B3 (5′-CGCGGATCCCTCCCATGGATGGAACGCCCGAATA) and cloned into the *Bam*HI site of pSWkanBH. A 1.7-kb *Pst*I fragment containing a Gm marker from pUC7Gm (a gift from S. Lory, University of Washington, Seattle) was cloned into the *Pst*I site of pSW906 to create pSW906KO. pSW906KO was mobilized from *E. coli* S17-1 into SW658 to generate cointegrate strains SW129 and SW323. A complementing plasmid containing the PA906 gene was made by amplifying a 2.65-kb product from PAO1SR with primers MER906B2 and 5′-CGCGGATCCCGATGGCCTTCTTCGAGGACAATGCAG. The product was digested with *Bam*HI and cloned into the *Bam*HI site of pBBR1MCS (14) to create pSW2.6. pBBR1MCS and pSW2.6 were each mobilized from *E. coli* SM10 λ pir into SW129 and SW323. PCR analysis of the Suc^r isolates from complemented cointegrate strains (see Table 2) with primers MER906G1 (5′-CACATCTTCATCGAGGAACTGCGCGCCTT) and MER906G4 (5′-GTGAAGGATTGGATGTATGGATCATTGG) yielded PCR products of ≈5.8-kb, which correlated with gene replacement of PA906 (no disruption would give rise to a predicted 4.2-kb PCR product). PCR analysis of the Suc^r isolates from cointegrates carrying pBBR1MCS (see Table 2) with primers MER906S4 (5′-CGCGGATCCGCAGCCGGGACCCGCATTTCATGC) and MER906S5 (5′-CGCGGATCCTCAGTCTTCGCGAGGCTTCTTCGCCGC) yielded a product size of ≈480-bp, indicating presence of the wild-type PA906 gene.

Transposon Mutagenesis in *P. aeruginosa* and Genetic Footprinting. A library of transposon insertion mutants (≈10^6) in *P. aeruginosa* was generated by mobilizing pFAC from the *E. coli* SM10 λ pir (≈10^{10}) into PAO1SR (≈10^9). After mating for ≈5 h at 37°C, transconjugants were selected for growth on LB medium containing Sm and Gm. Colony PCR with a transposon-specific primer, MarIN (5′-TACGTAACAGGTTGGCTGATAAGTCG), was performed on several Gm-resistant transconjugants, and the PCR products were sequenced to verify transposon insertions. For the *pyrF* genetic footprint analyses, ≈10^6 colony-forming units of the transposon insertion library was plated onto each of three selection conditions: (*i*) LB agar with Ura, Sm, and Gm; (*ii*) minimal agar with Sm and Gm; and (*iii*) LB agar with Ura, FOA, Sm, and Gm. Genomic DNA was isolated from the pool of insertion mutants from each selection condition and used as template in PCR for genetic footprinting (15). PCR was performed with a 6-carboxyfluorescein-labeled transposon-specific primer, MarOUT (5′-CCGGGGACTTATCAGCCAACC), and a chromosomal-specific primer, MERF6 (5′-AGGCTTCCAGGGTGTTCAGCATCCC), in the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s and 68°C for 6 min with 15 s added to the extension time for each cycle. PCR products were size-fractionated on a 7% denaturing polyacrylamide gel and analyzed on an ABI377 sequencer with GENESCAN DNA fragment analysis software. GENESCAN-2500 TAMRA (Applied Biosystems) was used as size standards. We were able to read consistently at least 1–1.5 kb with a resolution ranging from ±1 bp for small fragments (<500 bp) to ±30 bp for larger fragments. To footprint the PA1655, PA1654, and PA906 regions, PCR was performed with fluorescein-labeled MarOUT primer and chromosomal primers MERPA1655 (5′-CGCGGATCCTGGCAAGGCCCTGTCGCCGTAGA), MERPA1654, MER906S1 (5′-TGGCCTTCAAGGTGCTGGATTCGGAT), and MER906G1 with genomic DNA from ≈10^6 transposon insertion mutants selected on LB medium. PCR products were analyzed as described above.

Allelic Exchange in *P. aeruginosa*. For allelic replacement of *pyrF*, pSWΔFGm was mobilized from *E. coli* S17-1 into SWSce or SW658. After overnight mating at 37°C, transconjugants were plated onto minimal medium containing Sm, Gm, and Cb with and without Ura. To deliver transposon mutagenized regions of cloned *P. aeruginosa* DNA into the genome, *in vitro* transposition reactions with target plasmids pSW1654–55 and pSW906 were performed by using purified *Himar1* transposase as described (16). The pool of *in vitro* mutagenized plasmids was electroporated into the *E. coli* S17-1 and selected on LB containing Gm and Km. This protocol generated libraries representing ≈10^4 and ≈0.5×10^3 different transposon insertion events in the plasmids pSW1654–55 and pSW906, respectively. Approximately 10^8 *E. coli* donors carrying the *in vitro* mutagenized plasmid pSW1654–55 (represents a 1,000-fold excess in the number of different insertion events) were mated en masse with ≈10^8 SWSce recipients overnight at 37°C. Transconjugants were plated onto LB agar containing Gm and Cb. For analysis of the PA906 region, ≈10^9 *E. coli* donors containing mutagenized plasmid pSW906 (represents at least a 1,000-fold excess in the number of different insertion events) were mated with ≈10^9 recipients (SWSce or complemented strain SWSce2.6) for 5 h at 37°C. Transconjugants were plated onto LB agar containing Sm, Gm, and Cb or Sm, Gm, Cb, and Cm. Transposon insertion mutants were pooled and either were diluted to OD_{600} of ≈0.1 or had their genomic DNA isolated for genetic footprint analyses.

Results

***In Vivo* Transposition in *P. aeruginosa*.** The successful development of *in vivo* mariner transposon mutagenesis systems in *E. coli* and *Mycobacterium smegmatis* indicated the likelihood of this transposon working in any bacterium expressing the transposase (8). To determine whether the *in vivo* mariner transposition system could also produce high-density insertions in *P. aeruginosa*, the suicide delivery plasmid pFAC encoding the *mariner* transposase was transferred conjugally from *E. coli* into PAO1SR. We obtained a transposon insertion mutant library of ≈10^6 colony-forming units, similar to the transposition efficiency seen in *E. coli*, with an estimated frequency of obtaining a transposon insertion mutant of 1 per 200 recipients in *P. aeruginosa*.

Genetic Footprint of the *pyrF* Locus. Because *in vivo* mariner transposition is highly efficient in *P. aeruginosa*, we tested whether it was feasible to analyze genes functionally at the genomic scale. To develop PCR conditions for genetic footprint analyses in *P. aeruginosa*, the *pyrF* locus was chosen as a test region, because the *pyrF* gene product, orotidine-5′-monophos-

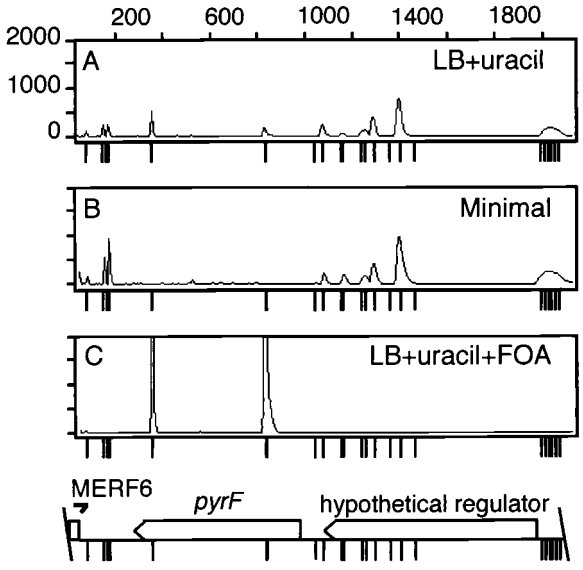

Fig. 1. Genetic footprinting at the *pyrF* locus. Locations of transposon insertions at the *pyrF* region were mapped by PCR with a fluorescent-labeled transposon-specific primer, MarOUT, and a chromosomal primer, MERF6. GENESCAN software quantifies the DNA fragments and displays the results as an electropherogram that shows fluorescence intensity (*y* axis) as a function of time, which corresponds to the molecular weight of the PCR products (*x* axis). PCR analysis of the *pyrF* locus from transposon insertion mutants isolated under three selective conditions: LB plates containing Ura (*A*), minimal plates (*B*), and LB plates containing Ura and FOA (*C*). Tick marks below each electropherogram and the corresponding genomic map of the *pyrF* region indicate the position of the TA dinucleotides. The two peaks within *pyrF* are off scale (*C*), because they are ≈4-fold more intense than the next largest peak seen in *A* and *B*.

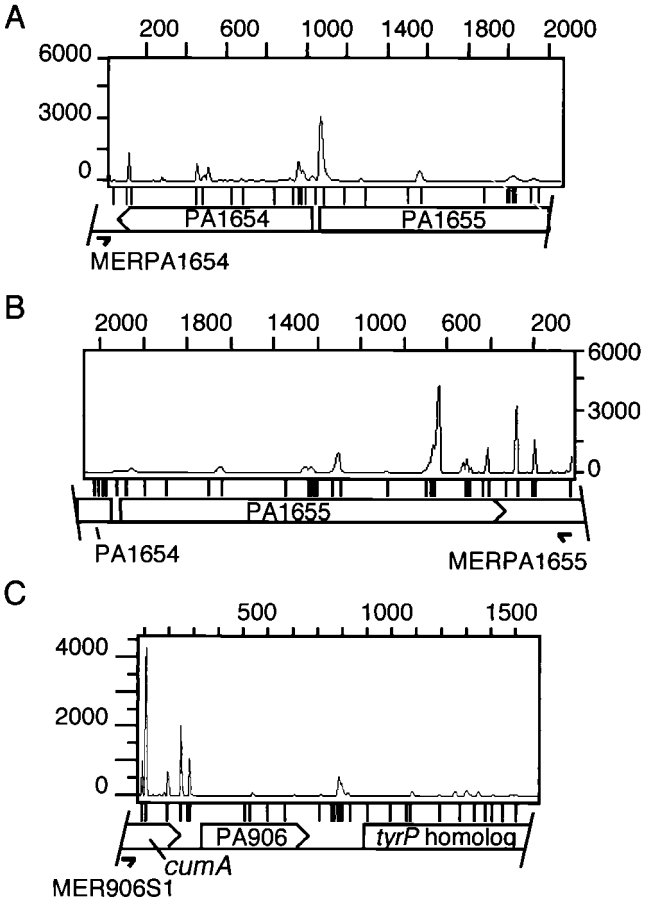

Fig. 2. Genetic footprinting of three *H. influenzae* essential gene homologs in *P. aeruginosa*. (*A*) PCR analyses of the PA1654 and PA1655 regions with primer MarOUT and chromosomal primers MERPA1654 (located 66 bp from the 3′ end of the PA1654 ORF) and (*B*) MERPA1655 (326 bp from the 3′ end of the PA1655 ORF). (*C*) PCR analysis at the PA906 locus with primer MarOUT and chromosomal primer MER906S1 (286 bp 5′ of the putative start site of PA906). Tick marks indicate the position of TA dinucleotides.

phate decarboxylase, which is required for biosynthesis of Ura (17), provides both positive and negative selection. *pyrF* mutants can be obtained on medium containing Ura or by plating on medium containing Ura and the pyrimidine analog FOA. The decarboxylase enzyme converts FOA to a toxic product; thus, *pyrF* mutants are resistant to FOA and will grow normally (18). Approximately 10^6 colony-forming units of the *mariner* transposon insertion library was plated onto each of three selection conditions: LB with Ura, Sm, and Gm; M9 minimal medium with Sm and Gm; and LB with Ura, FOA, Sm, and Gm. Mutants were pooled from each condition, and genomic DNA was isolated and used as template in PCR with fluorescent-labeled MarOUT and a chromosomal primer, MERF6, located 251-bp from the 3′ end of the *pyrF* gene. Fig. 1 shows PCR analyses of insertions in the *pyrF* locus with GENESCAN software. On LB medium supplemented with Ura, we were able to detect transposon insertions in at least two of three TA dinucleotides in *pyrF* (insertion between the two adjacent TA dinucleotides located at the 5′ end of *pyrF* cannot be distinguished; Fig. 1*A*). We found insertions in at least 11 of 17 possible TA dinucleotide insertion sites within a 1.5-kb region with no insertions identified in other dinucleotides. This result demonstrates that the *mariner* transposon achieves a high degree of saturation of target sites and seems to maintain the same site specificity seen *in vitro* and *in vivo* for eukaryotes and bacteria (8). In the absence of Ura in minimal medium, no PCR products corresponding to insertions within *pyrF* were detected (Fig. 1*B*). In contrast, footprints of the regions flanking *pyrF* seem to be similar in both rich LB (Fig. 1*A*) and minimal media (Fig. 1*B*). Selection with FOA yielded the expected insertions at TA dinucleotide sites exclusively within *pyrF* (Fig. 1*C*). PCR analyses with a second primer located 147-bp downstream from primer MERF6 showed the predicted shift in the pattern of TA insertions by ≈150 bp under LB, minimal, and FOA selection conditions (data not shown). We also verified that the PCR products were derived from the *pyrF* region by Southern blot analysis (data not shown), confirming that genetic footprinting results accurately reflect the composition of the mutant pool.

Genetic Footprint of *H. influenzae* Essential Gene Homologs in *P. aeruginosa*. A BLAST search (19) against available microbial genome sequences revealed that a number of essential genes identified in *H. influenzae* by using GAMBIT were conserved hypothetical genes in a wide variety of bacteria. To determine whether the homologs of three essential *H. influenzae* genes, HI1655 (putative lipoprotein), HI1654 (putative methyl transferase), and HI0906 (putative cytosine deaminase), were also essential in *P. aeruginosa*, we obtained genetic footprint data from these regions in *P. aeruginosa* (termed PA1655, PA1654, and PA906, respectively). PCR analysis was performed on genomic DNA from ≈10^6 colony-forming units of the *mariner* transposon insertion library selected on LB medium with Sm and Gm. We found insertions in at least 5 of 11 possible TA dinucleotides within the PA1654 ORF (Fig. 2*A*) and insertions in at least 8 of 21 possible TA dinucleotides within the PA1655 ORF (Fig. 2 *A* and *B*). A second and third primer located 129 bp 5′ and 66 bp 3′ with respect to primer MERPA1654 showed the

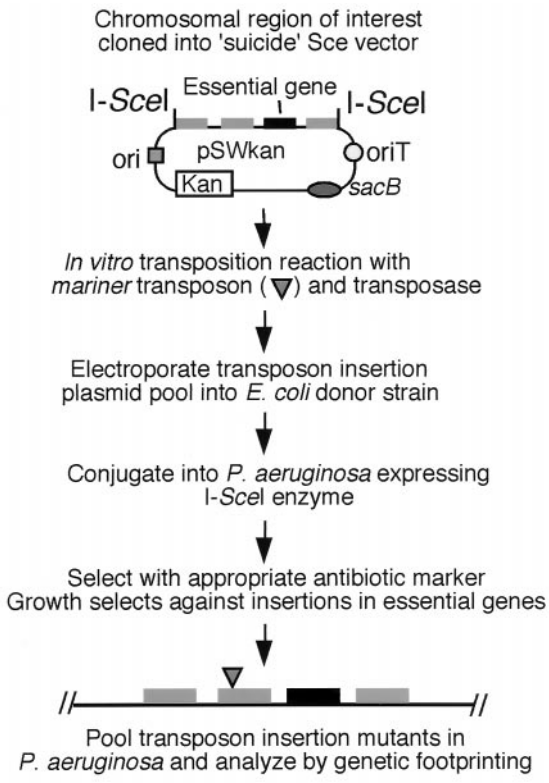

Fig. 3. SCE jumping in *P. aeruginosa*. Schematic diagram illustrates high-density mutagenesis of a targeted chromosomal region. Cloned regions of interest are mutagenized *in vitro* and introduced into *P. aeruginosa*. Presence of the I-*Sce*I enzyme prevents cointegrate formation. The mating mixture is plated onto the appropriate antibiotics, and growth selects against insertions in regions containing essential genes.

predicted electrophoretic mobility shift in the pattern of PCR products (data not shown). Our analysis showed that the PA1654 and PA1655 genes were nonessential for *in vitro* growth in *P. aeruginosa*. Fig. 2*C* shows genetic footprint results for the PA906 region in which at least 10 of 28 possible TA dinucleotides contained insertions within a 1.6-kb region flanking the gene. There were no significant PCR product peaks mapping to insertions at any of the four TA dinucleotides within the PA906 ORF. Lack of insertions within PA906 suggested that this gene is required for growth of *P. aeruginosa* on rich medium.

Development of an Allelic Exchange System in *P. aeruginosa*. The utility of high-density insertional mutagenesis of discrete (≈10-kb) chromosomal regions has been demonstrated recently for functional genomic studies of naturally transformable organisms (4). We sought to develop a genetic system for rapidly delivering transposon insertions to discrete regions of the chromosome in *P. aeruginosa* that would also allow recovery of specific mutants for further analysis. Unlike *H. influenzae*, *P. aeruginosa* is not able to take up naked DNA for chromosomal integration, and conjugation is commonly used to deliver DNA into this bacterium. However, conjugation does not favor gene replacement events and often results in the formation of cointegrates. We exploited the use of a rare cutting restriction endonuclease, I-*Sce*I, encoded by the mobile group I intron of the large 21S rRNA from *Saccharomyces cerevisiae* (20, 21). The recognition site of I-*Sce*I (18-bp) has not been reported in bacterial genomes to date. This enzyme allows a fast and efficient allelic exchange procedure, termed "SCE jumping" (see Fig. 3).

To develop SCE jumping in *P. aeruginosa*, the *pyrF* locus was chosen as a test gene for allelic exchange (Table 1). In the presence of the I-*Sce*I enzyme in minimal medium, Sm^r, Gm^r, and Cb^r isolates were obtained only when supplemented with Ura. In the presence of Ura, targeted knockout of the *pyrF* gene in SWSce resulted in gene replacement at a frequency of 100%, because 28 of 28 isolates screened were Suc^r and FOA^r. However, in the absence of Ura, we see a 4-log decrease in the frequency of obtaining transconjugants in SWSce. In the control recipient strain SW658, Sm^r, Gm^r, and Cb^r isolates were obtained with similar frequencies in minimal medium with and without Ura. In the absence of Ura, the frequency of a *pyrF* gene replacement was 0%. Of 25 isolates screened, 25 were Suc-sensitive (Suc^s) and FOA^s, indicating integration of the delivery plasmid into the chromosome. However, in the presence of Ura, the frequency of a *pyrF* gene replacement was ≈50%. Of 25 isolates screened, 12 were Suc^r and FOA^r, whereas the remaining 13 were Suc^s and FOA^s. These results demonstrate that SCE jumping is highly efficient in facilitating allelic exchange in *P. aeruginosa*.

Functional Analyses of Targeted Regions of the *P. aeruginosa* Chromosome. We used SCE jumping (Fig. 3) to create mutant pools containing random transposon insertions in cloned regions containing PA1654, PA1655, and PA906. For analyses of the PA1654 and PA1655 regions, 84 Gm^r and Cb^r transposon insertion mutants were pooled, and an aliquot was used for PCR analysis with primer MarOUT and chromosomal primer MERPA1654 located near the 3′ end of the PA1654 ORF. Fig. 4*A* shows the agarose gel electrophoresis of the PCR products obtained from the PA1654/PA1655 region. The distribution of insertions in PA1654 (PCR products mapping to the center of the gene) and PA1655 (intense PCR products mapping to the 5′ end of the gene) correlated with the pattern and fluorescence intensity of the PCR products seen in the GENESCAN results (Fig. 2 *A* and *B*). PCR analysis on the same pool of mutants with a second set of primers, MarOUT, and a chromosomal primer located 84-bp downstream of the PA1655 ORF yielded a PCR pattern consistent with the patterns seen in Figs. 2*B* and 4*A* (data not shown).

To analyze the PA906 region, 308 Sm^r, Gm^r, and Cb^r colonies were pooled, and genomic DNA was isolated for PCR analysis with primer MarOUT and chromosomal primer MER906G located ≈2 kb upstream of the PA906 ORF. Fig. 4*B* (lane 1)

Table 1. Allelic replacement of *pyrF* by using SCE jumping

Recipient strain	−Ura in minimal		+Ura in minimal	
	$Sm^rGm^rCb^r$	Suc^rFOA^r	$Sm^rGm^rCb^r$	Suc^rFOA^r
SW658	1.7×10^4	0/25	3.4×10^4	12/25
SWSce	0	0	0.9×10^4	28/28

Suicide plasmid pSWΔFGm was transferred conjugally from *E. coli* donor into *P. aeruginosa* recipients SWSce (contains I-*Sce*I-expressing plasmid) or SW658 (contains parent plasmid pJB658). After overnight mating at 37°C, transconjugants were plated onto minimal medium containing Sm, Gm, and Cb with or without Ura. Data are recorded as number of Sm^r, Gm^r, and Cb^r isolates per 10^8 recipients. Representative isolates were tested for Suc and FOA sensitivity.

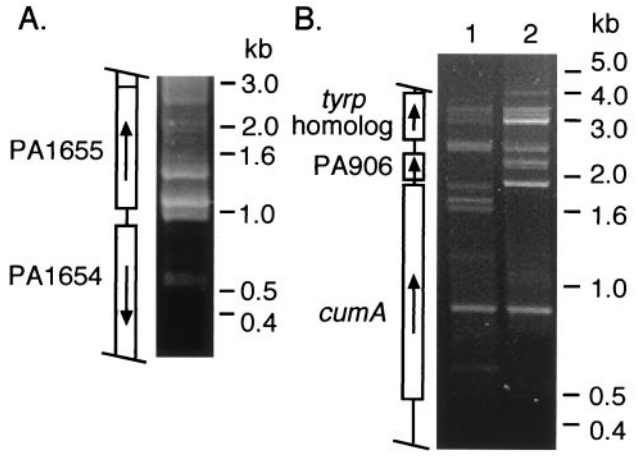

Fig. 4. Genetic footprinting at three targeted regions of the chromosome. Plasmid libraries of *in vitro* generated transposon insertions in pSW1654–55 and pSW906 were mobilized from *E. coli* to SWSce. After selection with the appropriate antibiotics, mutant pools were used for PCR analysis. Locations of the transposon insertions are aligned with the corresponding gene position. Arrows indicate direction of transcription. (*A*) Genetic footprinting at the PA1655 locus and part of the PA1654 gene. Colony PCR was performed with primers MarOUT and MERPA1654 located near the 3′ end of PA1654 on mutant pools. (*B*) Genetic footprinting at the PA906 region. PCR analysis was performed with primers MarOUT and MER906G1 located ≈2.0-kb from the 5′ end of the putative start site for PA906 (lane 1) on genomic DNA isolated from mutant pools. The inferred putative start site is based on the sequence of the PA906 homolog, *cumB* from *Pseudomonas putida* (26). The same pSW906 mutagenized plasmid library was introduced into strain SWSce2.6 complemented with a wild-type copy of PA906. Genomic DNA from mutant pools was used as template in PCR with primers MarOUT and MER906G1 (lane 2).

shows the agarose gel electrophoresis of the PCR products obtained from the PA906 region. We observed insertions at TA dinucleotides spanning the regions encoding *cumA* and the *tyrP* homolog as well as within the intergenic regions. No visible insertions were detected within the PA906 ORF, consistent with the whole-genome genetic footprint results in Fig. 2*C*. However, in a strain carrying the wild-type copy of PA906 on a complementing plasmid, transposon insertions from a pool of 102 Sm^r, Gm^r, Cb^r, and Cm^r mutants were observed in at least two TA dinucleotides sites within the chromosomal PA906 allele (Fig. 4*B*, lane 2). Thus, disruption of PA906 was possible only in the presence of the complementing plasmid.

Targeted Knockout with SCE Jumping. To verify the functional analyses of genes PA1655, PA1654, and PA906, insertional mutagenesis frequencies in each respective gene were determined. We used SCE jumping for allelic exchange with constructs pSWΔ1655Gm, pSWΔ1654Gm, and pSW906KO designed to produce site-directed knockouts of PA1655, PA1654, and PA906, respectively. The Km marker and the *sacB* gene on the constructs provide convenient assays for plasmid integration or gene replacement events. Expression of the *sacB* gene product renders the cell sensitive to growth on Suc, providing selection against cointegrates (22). Representative Gm^r and Cb^r transconjugants from each mating were tested for their ability to grow on Suc and Km. Targeted disruption of the PA1654 and PA1655 genes in strain SWSce resulted in gene replacement at a frequency of 100% for both PA1655 (87 of 87 isolates screened) and PA1654 (122 of 122 isolates screened). All of these colonies were Suc^r and Km^s. However, in the control strain SW658, the frequency of a gene replacement of PA1655 (32 of 49 screened were Suc^r and Km^s) and PA1654 (19 of 54 screened were Suc^r and Km^s) was 35% and 65%, respectively. The remaining isolates analyzed in this group were Km^r and Suc^s, consistent with plasmid integration in the chromosome.

Table 2. Frequency of Suc^r isolates from plasmid cointegrates

Cointegrate strain	Frequency of sucrose resistance
SW129 (pBBR1MCS)	1.9×10^{-6}
SW323 (pBBR1MCS)	1.0×10^{-6}
SW129 (pSW2.6)	2.2×10^{-4}
SW323 (pSW2.6)	1.8×10^{-4}

Complementing plasmid pSW2.6 containing PA906 and parent plasmid pBBR1MCS were mated into cointegrates SW129 and SW323 that contain plasmid pSW906KO integrated into the chromosome. Resultant Cb^r Gm^r, Km^r, and Cm^r isolates plus and minus complementation were grown overnight at 37°C in LB broth containing Gm and Cm without Km. Dilutions were plated onto LB agar containing Gm and Cm with and without 5% (vol/vol) sucrose. Frequency of sucrose resistance is calculated as the ratio of the number of isolates on agar containing 5% (vol/vol) sucrose to the number of isolates on agar without sucrose.

Site-directed knockout of PA906 yielded no transconjugants that were Gm^r, Cb^r, and Suc^r from a mating input of $\approx 10^8$ SWSce recipients. This result was consistent with the SCE jumping data in Fig. 4*B*, indicating that PA906 is essential for growth on rich medium. In the control strain SW658, we also did not obtain transconjugants containing a gene replacement of PA906. Of the 150 Gm^r and Cb^r isolates screened, all were Km^r and Suc^s, consistent with plasmid integration in the chromosome. However, by using recipient strain SWSce2.6, which carries a complementing plasmid, pSW2.6 containing a wild-type copy of PA906, we were able to obtain gene replacement of PA906 in the chromosome. There were 24 transconjugants obtained from mating of pSW906KO into SWSce2.6. PCR analysis was performed on two of these isolates with chromosomal primers (sequences are not located in the complementing plasmid or suicide delivery plasmid), which indicated disruption in the chromosomal copy of PA906 (data not shown).

Gene Replacement with *sacB* as a Counterselectable Marker. We next evaluated the essentiality of the PA906 gene by a method independent of the SCE jumping approach. We examined whether the *sacB* counterselectable marker could be used to promote a gene replacement event in PA906 in two independent cointegrate strains carrying either the complementing plasmid pSW2.6 or the parent plasmid pBBR1MCS. PCR analysis indicated that cointegrates SW129 and SW323 contain a plasmid integration of pSW906KO in the chromosome (data not shown). Table 2 shows the frequency of obtaining Suc^r isolates in the presence and absence of the complementing plasmid in LB medium with and without 5% (vol/vol) Suc. In the presence of the complementing plasmid, the frequency of obtaining Suc^r isolates was $\approx 10^{-4}$ for both cointegrates. In contrast, the frequency of obtaining Suc^r isolates was 2-log lower ($\approx 10^{-6}$) for both cointegrates in the absence of the complementing plasmid. PCR analysis with chromosomal primers that do not contain sequences within the suicide delivery plasmid pSW906KO or in the complementing plasmid pSW2.6 (see *Materials and Methods*) was performed on 16 Gm, Cm, and Suc^r representative isolates from each of the two plasmid-carrying strains. The results showed that gene replacement of PA906 occurred only in strains that carried the complementing plasmid pSW2.6 and not in those carrying pBBRIMCS (data not shown). In conclusion, three independent strategies indicate that the PA906 gene cannot be disrupted, unless complemented, demonstrating that PA906 is required for growth in *P. aeruginosa*.

Discussion

In this report, a complex *mariner* transposon insertion library generated *in vivo* in *P. aeruginosa* facilitated the used of a genetic

footprinting strategy (15) to assess the biological roles of three genes of unknown function. We also developed a simple and efficient allelic exchange system (SCE jumping) by using *in vitro* generated *mariner* mutagenesis to create high-density insertions within targeted chromosomal regions. The *sacB* gene has been traditionally used as a counterselectable marker to facilitate homologous recombination; however, the *sacB* marker is not suitable for our approach, because the transposon would efficiently disrupt this gene during *in vitro* mutagenesis. Expression of the I-*Sce*I enzyme in *P. aeruginosa* was shown to be extremely effective in promoting gene replacement events and selecting against cointegrates. In *P. aeruginosa*, we do not know the precise mechanism of I-*Sce*I enzyme-mediated homologous recombination. Double strand breaks induced by the I-*Sce*I enzyme *in vivo* have been shown to promote homologous recombination in eukaryotic and prokaryotic systems (10, 23–25) and have proven useful in strategies for gene replacement. SCE jumping in *P. aeruginosa* allows the recovery of a pool of mutants containing transposon insertions in specific chromosomal regions. Location of the insertions can be determined easily in each mutant by PCR and should prove useful in generating targeted mutations in any bacterium expressing the I-*Sce*I enzyme.

We exploited knowledge gained from studies in *H. influenzae* (4) to determine whether the homologs of three putative essential *H. influenzae* genes were also essential in *P. aeruginosa*. Genetic footprint analyses of the respective homologs PA1654, PA1655, and PA906 in *P. aeruginosa* showed that PA1654 and PA1655 were not essential under the *in vitro* conditions tested. In contrast, we did not detect insertions within the PA906 ORF. SCE jumping/genetic footprinting analysis and gene replacement strategies either by targeted disruption or by *sacB*/Suc counterselection have all indicated that PA906 is required for *in vitro* growth in *P. aeruginosa*. However, the HI0906 homologs in three other bacteria are not completely essential. A mutation of the HI0906 homologs (also of unknown function), *cumB* in *P. putida* (26), and *orf74* in *Bradyrhizobium japonicum* (27) resulted in an extended lag phase in both organisms. In contrast, a mutation in the HI0906 homolog in *E. coli orf178* (encoding a putative membrane protein involved in cell killing with the *gef* family of toxic proteins) confers no reported growth defect (28). Essentiality of a gene may vary among bacterial species, because some gene products may fulfill different roles required by an individual bacterium. For instance, the ferric-uptake regulator *fur* is essential in *P. aeruginosa* but not in several other bacteria, including *E. coli* (29). Because we were not able to obtain a PA906 mutant in *P. aeruginosa*, it is possible that the requirement for the PA906 gene product for optimal growth is greater in *P. aeruginosa* than for the respective homologs in *P. putida* and *B. japonicum*. The fact that both *cumB* and *orf74* mutants have growth defects and our inability to isolate mutations in PA906 despite strong selection are consistent with the essentiality of HI0906 in *H. influenzae*.

Our results indicate that one of three *H. influenzae* essential gene homologs seems to be needed for growth in *P. aeruginosa*. Because the *P. aeruginosa* genome (≈6 megabases) is ≈70% greater in size than the *H. influenzae* genome (≈1.8 megabases), it is not surprising that a number of essential genes in *H. influenzae* were not essential in *P. aeruginosa*. HI1654 has been proposed to be part of the minimal gene set essential for cellular function based on conserved sequences between *Mycoplasma genitalium*, the smallest genome sequenced (≈0.6 megabases), and *H. influenzae*, which has a genome ≈3 times greater than *M. genitalium* (30). The HI1654 homolog was also absent in the list of nonessential genes in both *M. genitalium* and *Mycoplasma pneumoniae* (31). It is probable that the larger coding capability of *P. aeruginosa* compared with organisms with smaller genomes corresponds to an increase in redundant gene functions. A larger comparative genomic scale analysis of *H. influenzae* essential gene homologs in *P. aeruginosa* will be required to assess fully the correlation between genome size to the proportion of genes in the chromosome needed for viability.

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