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The Use of Oligonucleotide Recombination to Generate Isogenic Mutants of Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract

Oligonucleotide recombination allows the introduction of specific point mutations into the bacterial genome. Here we report the successful application of this technique to clinical isolates of *Pseudomonas aeruginosa* to allow subsequent investigations of the biological impact associated with resistance-conferring mutations.

Keywords

Recombination; *Pseudomonas aeruginosa*; fluoroquinolones; resistance mutations

Homologous recombination is a highly conserved process that facilitates genetic exchange between identical or nearly identical DNA molecules (Lovett et al., 2002). Swingle et al. (Swingle et al., 2010) demonstrated that bacterial recombination can be achieved experimentally by introducing high concentrations of single stranded synthetic oligonucleotides via electroporation. The authors successfully utilized this procedure to introduce site-specific mutations into the genomes of Pseudomonas syringae, Shigella flexneri, Escherichia coli, and *Salmonella typhimurium*. In the present study, we describe the use of this procedure to generate isogenic mutants from clinical isolates of *Pseudomonas aeruginosa*, an organism in which this technique has not been attempted previously. *Pseudomonas aeruginosa* is a leading cause of infection in the hospital setting, and resistance to the fluoroquinolone antibiotics has risen dramatically with their increase in use. (Linder et al., 2005, Neuhauser et al., 2003).

We recently showed in a large collection of clinical isolates that strains containing the gene that encodes for the exotoxin ExoU more readily acquire a second mutation in *parC* compared to *exoS+* strains in addition to the fluoroquinolone resistance-conferring mutation in *gyrA* (Agnello and Wong-Beringer, 2012). Therefore, we sought to understand the biological effects of this specific point mutation in both *exoU* and *exoS* clinical isolates by

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utilizing the technique of oligonucleotide recombination to create isogenic *parC* mutants for multiple *exoU* and *exoS* clinical isolates.

Pseudomonas aeruginosa isolates were obtained from the respiratory tract of hospitalized patients with pneumonia and were stored at −80°C in 70% glycerol until ready for use. Seven isolates *(3 exoU+* and 4 *exoS+)* were selected for recombination from our previous collection of 270 clinical isolates (Agnello and Wong-Beringer, 2012) based on the presence of the *exoU* or *exoS* gene and a mutation in *gyrA* conferring fluoroquinolone resistance. Our goal was to generate isogenic double mutants from these clinical strains. Oligos 22 nucleotides and 60 nucleotides in length were used and were designed based on the *parC* gene sequence of strain PAO1 (Winsor et al., 2011) (Table 2). The oligos were identical to the PAO1 sequence from nucleotides 249 to 270 and 230 to 289 respectively, save for the point mutation TCG→TTG at locations 12 and 31 of the oligos, corresponding to nucleotide 260 in the *parC* gene (Figure 1). This point mutation, which is the most common *parC* mutation observed among fluoroquinolone-resistant clinical strains, gives rise to the Ser87→Leu amino acid change in the ParC protein.

For electroporation, a protocol for *P. aeruginosa* was adapted (Choi et al., 2006). An overnight culture was diluted 100 fold, incubated with shaking at 37°C and harvested at OD_{600}= 0.3-0.5 by centrifugation at 3200 × *g*. Cells were resuspended twice in 300 mM sucrose at room temperature. Either the 22-nt or the 60 nt oligo (5-6 μg in 2-3 μl) was added to 40 μl of electrocompetent cells and transformed by electroporation at 2.5 kV in a .2 cm cuvette using a Micropulser (Bio-Rad). SOC medium (1 ml) was added immediately and the cells were outgrown overnight at 37°C on *Pseudomonas* Isolation Agar plus levofloxacin at concentrations 2, 4, 8, and 16 fold above the original minimum inhibitory concentration (MIC) of the isolates. For each isolate, a mock experiment was performed as a control in which cells underwent electroporation without the addition of any oligo.

Strains electroporated with the oligo 22 nucleotides in length did not grow on any selection plates. “Mock” control strains did not grow on any selection plate in 4 out of the 7 experiments. In the other 3 experiments, the mock cultures were able to grow on plates containing levofloxacin at a concentration 2 times higher than the strain’s MIC; however, subsequent sequencing of these colonies showed that no change had occurred in the *parC* gene, demonstrating that growth of the control strains on selection was not due to the presence of a spontaneous target site mutation.

Strains electroporated with the 60 nucleotide oligo grew on selection plates with a concentration of up to 16 times higher than their original MIC. Single colonies were selected from the highest concentration of levofloxacin-containing plates and used to inoculate 5 ml of LB broth, from which DNA was extracted using the DNeasy Mini Kit (Qiagen). Prior to sequencing, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Primers for PCR were previously published (Jalal and Wretlind, 1998) and are listed in Table 2. Sequencing results confirmed that all strains had incorporated the TCG→TTG mutation in the *parC* gene at nucleotide 260, and no extraneous recombination had occurred in the *gyrA* gene, despite the high degree of sequence similarity to *parC*.

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Minimum inhibitory concentration (MIC) to levofloxacin was measured by broth microdilution according to guidelines recommended by CLSI (CLSI), 2007). MIC was also measured with the addition of an efflux pump inhibitor (Phe-Arg β-naphthylamide dihydrochloride, Sigma) at 20 μg/ml in order to more accurately reflect the resistance phenotype conferred by the specific point mutation introduced (Lomovskaya et al., 2001). The *parC* mutation increased the MIC in 5 out of the 7 isolates compared to the parent strains (Table 1).

To measure growth rates, overnight cultures were diluted 10 fold and grown at 37°C with shaking. Cultures (150 μl) were sampled every 30 min for 8 hours and turbidity was measured in triplicate at OD_{600} using a microplate reader (Tecan Group Ltd., Switzerland). Results are an average of at least 3 independent experiments. Generation time during exponential phase was calculated by dividing the time interval over the number of generations. Interestingly, the growth rates of the recombinants were not significantly affected; the average difference between the generation times of the recombinants compared to the parents was less than 1 hour (Table 1).

The technique of oligonucleotide recombination described here that we have adapted for use in clinical isolates of *Pseudomonas aeruginosa* is an efficient and practical approach for inserting point mutations into specific sections of the bacterial genome. The threat of widespread increasing antibiotic resistance makes it imperative to gain a full understanding of the biological impact resistance mutations can have on the individual organism as well as the bacterial population as a whole. As laboratory-derived mutants do not reflect real world pathogenesis (Fux et al., 2005), this technique can prove useful in generating isogenic mutant strains from clinical isolates for in depth investigations.

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Highlights

- We inserted specific point mutations into clinical isolates of *P. aeruginosa*.
- We adapted the method of oligonucleotide recombination for use in *P. aeruginosa*.
- We created isogenic, fluoroquinolone-resistant mutants from 7 clinical strains.

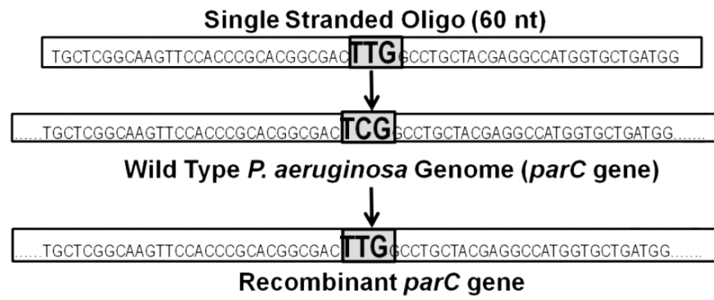

Figure 1. Schematic of Recombination

The successful single stranded oligonucleotide used was 60 base pairs in length and identical in sequence to a portion of the wild type *parC* gene, save for the point mutation indicated. The strains integrated the oligo into their genomes at the site of similarity within the *parC* gene.

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Table 1

Characteristics of Parent and Recombinant Strains

Parent Strain	TTSS Genotype (exoU/exoS)	MIC/MIC + EP1[a] (µg/ml)	Generation time[b] (hrs)	Recombinant	Recombinant MIC/MIC+EPI[a] (µg /ml)	Recombinant Generation time[b] (hrs)
U-1	*exoU*+	16/1	3.0	**U-1*parC****	16/1	2.2
U-2	*exoU*+	2/2	2.7	**U-2*parC****	16/2	2.6
U-3	*exoU*+	16/1	2.6	**U-3*parC****	16/1	3.0
S-1	*exoS*+	16/1	2.6	**S-1 *parC****	16/8	2.8
S-2	*exoS*+	16/1	1.9	**S-2*parC****	16/1	1.7
S-3	*exoS*+	8/1	3.4	**S-3*parC***	64/16	3.1
S-4	*exoS*+	2/.25	2.3	**S-4*parC****	8/4	3.1

[a]The MIC of levofloxacin was measured with the addition of an efflux pump inhibitor in order to remove the contribution of efflux pump overexpression to the resistance phenotype observed.

[b]Generation time is an average of 3 independent experiments and was determined by calculating the number of generations during exponential phase

Table 2

Oligos and Primers used

Name	Use	Primer Sequence (5'-3')	References
gyr A	PCR/ Sequencing	Forward: ttatgccatgagcgagctgggcaacgact Reverse: aaccgttgaccagcaggttgggaatctt	(Jalal and Wretlind, 1998)
par C	PCR/ Sequencing	Forward: cgagcaggcctatctgaactat Reverse: gaaggacttgggatcgtccgga	(Jalal and Wretlind, 1998)
parC*60	Recombination	tgctcggcaagttccacccgcacggcgacttggcctgctacgaggccatggtgctgatgg	Present study
parC*22	Recombination	gcacggcgacttggcctgctac	Present study

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