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Use of Polymerase Chain Reaction for the Determination of About 2.5 kb fpvA and fpvB Gene Sequences in Pseudomonas aeruginosa

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1. Introduction
Pseudomonas aeruginosa produces three different pyoverdines, types I-III (Cornelis et al., 1989), which are able to chelate iron and form ferripyoverdine complexes that are recognized and transported by different ferripyoverdine receptors present on the outer membrane. The ferripyoverdine receptor gene, fpvA of P. aeruginosa (PAO1) has been characterized previously (Poole et al., 1993). In addition, the other iron-repressible outer membrane receptor proteins for types II and III ferripyoverdine complexes were recently identified and characterized by cloning (De Chial et al., 2003). Following the observation that an fpvA mutant could demonstrate low ferripyoverdine uptake compared with wild type (Poole et al., 1991; Gensberg et al., 1992), an alternative ferripyoverdine receptor gene fpvB was identified and a fragment (562 bp) was amplified by polymerase chain reaction (Ghysels et al., 2004). In addition, the growth of several P. aeruginosa pyoverdine-negative mutants, found to inhabit the lungs of cystic fibrosis patients, were stimulated by existing pyoverdine types, providing additional confirmation for the existence of an alternative route for ferripyoverdine uptake (De Vos et al., 2001; Ghysels et al., 2004).

2. Experimental procedures
Bacterial strains used in this study.

The strains used in this study included reference and test strains and are as listed in Table 1.

Primers.
Several internal and external primers were designed from the fpvA (I-III) gene sequences of reference strains (PAO1, PA 14, ATCC 27853, 7NSK2, and 59.20) present in the

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Table 1. List of strains used in this study
database for the purpose of amplification and sequencing of the ferr ipyoverdine receptor (fpvA and fpvB) genes in the strains under study. The primer sequences are shown in Table 2.

DNA preparation method.
DNA was extracted from bacteria samples either by boiling in filter-sterilized water or using a PCR template preparation kit (Roche) according to the manufacturer's instructions.

Sequencing of PCR products.
All sequencing was performed at the VIB sequencing facility. Using the CAP2 software program (www.infobiogen.com), the resulting external forward and reverse sequences, in addition to the internal sequences for all test strains, were aligned, and the resulting consensus sequence was approximately 2.5 kb.

PCR conditions.
The various PCR mixes and cycling conditions are shown in Tables 3A-D.

3. Amplification of the fpvB gene (2.5 kb) in P. aeruginosa strains

List of bacterial strains used is shown in Table 1.

DNA preparation method.
Sterile colonies from 13 bacterial samples were grown on LB media overnight at 37°C and then boiled in 400 µL water at 95°C for 10 min. The resulting templates were used for PCR.
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Gel electrophoresis. Amplified PCR products were run on an agarose gel (0.8%) at 100 V for 70 min. Subsequently, the gel was stained in ethidium bromide for 12 min. Stained gels were visualized and illuminated under UV light (260 nm).

Sequencing of PCR products. Amplified DNA was purified using a Sigma Gen-Elute PCR clean up kit, and 100 ng/µl of purified DNA was sent for sequencing with 5 µM of each primer; these primers were used to sequence the external portions of the *fpvB* gene of nine *P. aeruginosa* strains as shown below:

Table 4. Primers used to sequence the external portions of the *fpvB* gene of nine *P. aeruginosa* strains

| Primer  | Position       |
|---------|----------------|
| FpVB1   | 803-820        |
| FpVB2   | 833-850        |
| FpVR1   | 1609-1628      |
| FpVR2   | 1631-1648      |

The resulting sequences were aligned with the sequence of the PAO1 *fpvB* gene using DNA-manager software. The alignment was performed for the PAO1 *fpvB* sequence plus all resulting forward sequences (for nine sequenced strains) and the PAO1 *fpvB* sequence plus all resulting reverse sequence (for nine sequenced strains).

The purpose of this alignment was to facilitate the design of internal primers for the sequencing of the internal portion of the *fpvB* gene, and two sets of internal primers were designed for this purpose as shown below:

- FpVB1 (forward) from position (803-820) of aligned forward sequences;
- FpVB2 (reverse) from position (833-850) of aligned forward sequences;
- FpVR1 (forward) from position (1609-1628) of aligned reverse sequences; and
- FpVR2 (reverse) from position (1631-1648) of aligned reverse sequences.

100 ng/µl of purified DNA plus 5 µM of each primer was used for sequencing the internal portion of the gene.

2.5 kb *fpvB* gene sequence for nine strains.

Using the CAP2 software program, the resulting external forward and reverse sequences, in addition to four internal sequences for each of the nine strains, were aligned; the resulting consensus sequence was approximately 2.5 kb.

Amplification of the *fpvA* gene (2.5 kb) in *P. aeruginosa* strains.

Amplification of the *fpvA* types I, IIa, IIb, and III genes in eight *P. aeruginosa* strains in which about 2.5 kb *fpvB* gene was previously amplified was performed using primers designed in this study (Table 2: List of primers).
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Gel electrophoresis. Amplified PCR products were run and processed as described above.

Sequencing of PCR products. Amplified DNA was purified as described above and 100 ng/µl of purified DNA was sequenced with 5 µM of each primer; these primers were used to sequence the external portions of the \textit{fpvA} gene of three \textit{P. aeruginosa} strains as shown below:

Table 6. Three \textit{P. aeruginosa} strains

The resulting sequences were aligned with the complete sequence of the \textit{fpvA} gene of three reference strains (PAO1, PA14, and 10-15) as described above. The alignment was done for the \textit{fpvA} sequences of three reference strains plus all resulting forward sequences (for three sequenced strains) and the \textit{fpvA} sequences of three reference strains plus all resulting reverse sequences (for three sequenced strains).

The purpose of this alignment was to facilitate the design of internal primers for the sequencing of the internal portion of the \textit{fpvA} gene, and two sets of internal primers were designed for this purpose as shown below:

- Int1AF1 (forward) from position (800- 818) of aligned forward sequences;
- Int1AR1 (Reverse) from position (860- 879) of aligned forward sequences;
- Int1BF1 (forward) from position (1632-1650) of aligned reverse sequences; and
- Int1BR1 (Reverse) from position (1718- 1736) of aligned reverse sequences.

100 ng/µl of purified DNA plus 5 µM of each internal primer was used to sequence the internal portion of the \textit{fpvA} gene.

\textit{fpvA} gene (2.5 kb) sequence for three strains. Using the CAP2 software program, the resulting external forward and reverse sequences, in addition to four internal sequences for each of the three strains, were aligned, and the resulting consensus sequence was approximately 2.5 kb.

PCR mix for \textit{fpvA} type IIa gene amplification using primers 2A-PF and 2A-PR was as in Table 3C, except that no MgCl$_2$ was used. PCR cycling conditions were as in Table 3B.

Gel electrophoresis was performed as described above.

Sequencing of PCR products. Amplified DNA was purified as described above and 100 ng/µl of purified DNA was sequenced with 3 µM of each primer and these primers were used to sequence the external portions of the \textit{fpvA} gene of \textit{P. aeruginosa} strain W15 Aug 15 as shown below:

Table 7. \textit{P. aeruginosa} strain W15 Aug 15

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The resulting sequence was aligned with the sequence of the \textit{fpvA} gene of five reference strains (7NSK2, ATCC 27853, MSH, 2-164, and 1-60) as described above. The alignment was done for the \textit{fpvA} gene sequences of five reference strains plus resulting forward sequences (for one sequenced strain) and the \textit{fpvA} gene sequences of five reference strains plus resulting reverse sequences (for one sequenced strain).

The purpose of this alignment was to facilitate the design of internal primers to enable the sequencing of the internal portion of the \textit{fpvA} gene; the internal primers designed are shown below:

- \textit{EFT-2A} (forward) from position (500-519) of aligned forward sequences;
- \textit{Int2AF2} (forward) from position (801-819) of aligned forward sequences;
- \textit{Int2AR2} (reverse) from position (880-898) of aligned forward sequences;
- \textit{Int2BF2} (forward) from position (1537-1554) of aligned reverse sequences;
- \textit{Int2BR2} (reverse) from position (1736-1754) of aligned reverse sequences;
- \textit{2A-Int2AF} (forward) from position (2344-2362) of aligned reverse sequences; and \textit{ERT-2A} (reverse) from position (2400-2419) of aligned reverse sequences.

100 ng/µl of purified DNA plus 5µM each of internal primers were used for sequencing the internal portion of the gene. The same procedure was followed for type 11b except for the external primers that differed (\textit{ExtF-2A} and \textit{ExtR-2A}) and only internal primers shown in bold above were used.

\textit{fpvA} gene (2.5 kb) sequence for two strains. Using the CAP2 software program, the resulting external forward and reverse sequences in addition to the four internal sequences for each of the two strains (seven internal sequences in the case of W15Aug15) were aligned and the resulting consensus sequence was approximately 2.5 kb.

PCR mix for \textit{fpvA} type III gene amplification using primers \textit{EFT-II1A} and \textit{ERT-II1A} is as shown in Table 3C. PCR cycling conditions are as shown in Table 3B. Gel electrophoresis was performed as described above. Sequencing of PCR products. Amplified DNA was purified as described above and 100 ng/µl of purified DNA was sequenced with 3 µM of each primer; these primers were used to sequence the external portions of the \textit{fpvA} gene of three \textit{P. aeruginosa} strains as shown below:

Table 8. Three \textit{P. aeruginosa} strains.

The resulting sequences were aligned with the sequence of the \textit{fpvA} gene of three reference strains (59.2O, ATCC 013, and 206-12) as described above. The alignment was done for the \textit{fpvA} sequences of three reference strains plus all resulting forward sequences (for three sequenced strains) and the \textit{fpvA} sequences of three reference strains plus all resulting reverse sequences (for three sequenced strains).
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The purpose of this alignment was to facilitate the design of internal primers for the sequencing of the internal portion of the fpvA gene; the internal primers (in bold) are shown below:

- Int3AF3 (forward) from position (940-958) of aligned forward sequences;
- Int3AR3 (Reverse) from position (975-992) of aligned forward sequences;
- Int3BF3 (forward) from position (1690-1709) of aligned reverse sequences;
- Int3-BF3 (forward) from position (1604-1622) of aligned forward sequences;
- Int3BR3 (Reverse) from position (1820-1839) of aligned reverse sequences;
- FpVAint3F (forward) from position (2219-2237); and
- FpVA-3PR (Reverse) from position (2542-2559).

100 ng/µl of purified DNA plus 3 µM (5µM in the case of int3AF3 and int3BR3) each of internal primers were used for sequencing the internal portion of the gene. Also, a new PCR amplification using primers int3-BF3 and FpVA-3PR was performed to enable sequencing of the end portion of the fpvA gene.

fpvA gene (2.5kb) sequence for 3 strains. Using the CAP2 software program, the resulting external forward and reverse sequences, in addition to five internal sequences for each of the three strains, were aligned; the resulting consensus sequence was approximately 2.5 kb.

Table 9. PCR amplification of fpvA gene (1.5 kb) in Pseudomonas strain Br678

Table 10. ClustalX alignment of fpvA sequences
Design of primers. Two sets of degenerate primers were designed in this study following a ClustalX alignment of the \textit{fpvA} sequences of 11 \textit{P. aeruginosa} strains as shown above. DNA was purified and prepared for PCR as described above. PCR cycling conditions were as described above except that the annealing temperature was increased to 57°C.

Gel electrophoresis. Gel electrophoresis of amplified DNA involved an application of 8 µL amplified PCR product and 2 µL loading dye on a 0.8% agarose gel in 1× TAE buffer and performed at 100 V for 65 min. Subsequently, the gel was stained in ethidium bromide for 12 min and illuminated under UV light.

Sequencing of PCR products. Amplified DNA was purified and sequenced as described above with 5µM each of primer; these primers were used to sequence the amplified purified PCR product of strain Br678. \textit{fpvA} gene (1.5 kb) sequence for strain Br678. Using the CAP2 software program, the resulting forward and reverse sequences were aligned, and the resulting consensus sequence was approximately 1.5 kb. The 1.5-kb \textit{fpvA} sequence of Br678 was 96% identical and 97% similar to \textit{fpvA} type II of \textit{P. aeruginosa} isolate 2-164 at nucleotide and amino acid levels, respectively.

4. Amplification of \textit{fpvB} gene (2.5 kb) in \textit{P. aeruginosa} strain Br678 DNA was purified and prepared for PCR as described above. PCR cycling conditions were as described above in Table 3B. Gel electrophoresis was as described above.

Sequencing of PCR products. Amplified DNA was purified and sequenced as described above with 5 µM of each primer (\textit{PA4168F} and \textit{PA4168R} as above); these primers were used to sequence the external portions of the \textit{fpvB} gene of strain Br678. In addition, the two sets of internal primers previously designed for the sequencing of the internal portion of the \textit{fpvB} gene were used to sequence the internal portion of the gene as shown below:

- \textit{FpVBF1} (forward) from position (803-820) of aligned forward sequences;
- \textit{FpVBF2} (Reverse) from position (833-850) of aligned forward sequences;
- \textit{FpVBR1} (forward) from position (1609-1628) of aligned reverse sequences; and
- \textit{FpVBR2} (Reverse) from position (1631-1648) of aligned reverse sequences.

100 ng/µl of purified DNA of filter sterilized water plus 5µM of each internal primer was used to sequence the internal portion of the gene.

5. \textit{fpvB} gene (2.5 kb) sequence for strain Br678 Using the CAP3 software program, the resulting external forward and reverse sequences, in addition to four internal sequences for strain Br678, were aligned, and the resulting consensus sequence was approximately 2.5 kb.

6. Results The nucleotide sequences of both \textit{FpvA} and \textit{FpvB} determined in this study have been deposited in the GenBank database (Bodilis et al., 2009).
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Table 11. External and internal primers used in this study.
Fig. 1. Homology trees (a-c) show the percent relatedness of the \textit{fpvA} and \textit{fpvB} genes in \textit{P. aeruginosa} strains Br678 and Mi162 (\textit{fpvA} gene sequence of strain Mi162; homology tree is shown in another manuscript in preparation) and other test strains to those of reference strains MSH (Smith et al., 2005), ATCC 27853, 1-60, 2-164 (Spencer et al., 2003), 7NSK2 (De Chial et al., 2003), and PAO1 (Stover et al., 2000). Trees were constructed using the DNA manager software following the alignment of all the nucleotide (\textit{fpvA} and \textit{fpvB}) sequences for the individual \textit{P. aeruginosa} test and reference strains.
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Fig. 2. Gel image of fpvB (2.5 kb) gene amplification in P. aeruginosa strains. Lanes 1-13: PAOI (positive control), ATCC 27853, 7NSK2, 59.20, W15 Aug 21, W15 Aug 16, W15 Aug 15, W15 Dec 11, W15 Dec 10, W15 Dec 9, W15 Dec 6, W15 Dec 1, and negative control (200-bp ladder).

Fig. 3. Gel image of fpvA (2.5 kb) gene amplification in fpvA type I P. aeruginosa strains. Lanes 2-5: PAOI (positive control), W15 Aug 21, W15 Dec 6, W15 Dec 1; negative control (lane L, 200-bp ladder).
Fig. 4. Gel image of \textit{fpvA} (2.5 kb) gene amplification in \textit{fpvA} type IIa \textit{P. aeruginosa} strains (a). Lanes 1-3: 7NSK2 (positive control), W15 Aug 15, and negative control; L, 200-bp ladder. Gel image of \textit{fpvA} (2.5 kb) gene amplification in \textit{fpvA} type IIb \textit{P. aeruginosa} strains (b). Lanes 1-3: ATCC 27853 (positive control), negative control, W15 Dec 11; L, 200-bp ladder.

Fig. 5. Gel images of \textit{fpvA} (2.0 and 1.0 kb) gene amplification in \textit{fpvA} type III \textit{P. aeruginosa} strains. Lanes 1-5 (a, Lanes 1-4): 59.2O (positive control), W15 Dec 9, W15 Dec 10, W15 Aug 16, and negative control (b); L, 200-bp ladder.
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Fig. 6. Gel images of fpvA and fpvB (2.5 kb) gene amplification in P. aeruginosa strain Br678. Bands correspond to fpvA and fpvB genes in test strain (Br678) and positive control (PAO1), respectively.

Fig. 7. Gel images of fpvA (500 bp) and fpvB (2.5 kb) gene amplification in P. aeruginosa strain Mi162. Bands correspond to fpvA (Lane 1; a) and fpvB (Lane 1 b) gene amplification in test strain (Mi162) and positive controls (PAO1 for fpvB (Lanes 2, Figure 7b) and ATCC 27853 for fpvA) (Lane 2, a).
8. Percent identity and similarity of fpvA and fpvB at the nucleotide and amino acid levels (BLAST search against the NCBI database)

Table 12. Percent identity at the nucleotide level for each individual test strain in relation to the reference strains.

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Table 13. Percent similarity of fpvA and fpvB at the nucleotide and amino acid levels (BLAST search against the NCBI database)

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Discussing the production of pyoverdine in P. aeruginosa under iron-limiting conditions. Pyoverdine is known to affect iron and contribute to the virulence of this organism. Studies have documented that pyoverdine can strip iron from transferrin (Cox, 1986) and produce pyocyanin, a virulence factor.

PCR methods are considered reliable and less time-intensive than cloning. Since PCR is not limited by pyoverdine production as is siderotyping, it is the preferred method for typing Gram-negative bacteria. PCR has been used extensively in studies involving the identification and characterization of P. aeruginosa.
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has provided for the first time a means to determine the
fpvA and fpvB gene sequences (~2.5 kb) in
P. aeruginosa
clinical and environmental isolates using experimental PCR.

Future perspective

Pseudomonas aeruginosa affects immunocompromised individuals like the AIDS patients
undergoing antiretroviral therapy, in these individuals, it has been documented that
P. aeruginosa causes a range of infections among which are urinary tract infections,
respiratory infections, gastrointestinal infections, bone and joint infections and bacteremia,
the case fatality rate in these patients is near 50% (Todar, K. 2004).

Pyoverdine the siderophore secreted by
P. aeruginosa is very important to it and siderophore biosynthesis has been documented to represent an attractive antibiotic target (Quadri, 2000).

fpvA has also been proposed to drive diversity at the pyoverdine locus (Smith et al., 2005),
looking at the strains I worked with, especially the clinical isolates (Isolated from burn
wound), the primers used for amplification were different from those used for the existing
fpvA type II pyoverdine isolates and following amplification and sequencing, a variant form
(different from the already existing ferripyoverdine receptor gene types) of the
ferripyoverdine receptor genes is presented (strain Br678, fpvA 93% identical to the
fpvA gene of other strains in the homology tree (see homology tree c); comparison based on the
sequenced 1.5 kb sized
fpvA gene, strain Mi162
fpvB 98% identical to the
fpvB gene of other
strains in the homology tree (see homology tree a); comparison based on the sequenced 2.5
kb sized
fpvB gene), this may then justify a correlation between amino acid sequence
diversity of immunogenic bacterial proteins and evasion of host immune defense
mechanisms (Tummler and Cornelis, 2005).

It would be interesting to study these strains in the future to pave way for the full
understanding of underlying mechanism of antibiotic resistance. More research would be
done in this regard hopefully.

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The main goal in compiling this book was to highlight the situation in Africa in terms of AIDS and opportunistic diseases. Several chapters reveal great poverty, an apocalyptic situation in many parts of Africa. Global migration of people resulted in their exposure to pathogens from all over the world. This fact has to be acknowledged and accepted as African reality. New, unconventional hypotheses, not determined by established dogmas, have been incorporated into the book, although they have not yet been sufficiently validated experimentally. It still applies that any dogma in any area of science, and medicine in particular, has and always will hinder progress. According to some biologists, in the future, AIDS is very likely to occur in a number of variations, as a direct result of the ongoing processes in the global human society. Thus, we urgently need a comprehensive solution for AIDS, in order to be ready to fight other, much more dangerous intruders.
