16

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

Julie Osaretin Osayande
Department of Molecular and Cellular Interactions (MINT)
Vrije Universiteit Brussels
Belgium

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).
PCR was developed in 1983 by Kary Mullis (Karry Mullis Nobel Lecture, December 8, 1993) and involves the selective amplification of specific regions of DNA for extensive use in molecular biology (Sambrook and Russell, 2001). Using primers designed in this study, the complete sequence of the ferripyoverdine receptor genes (*fpvA* and *fpvB*) from several *P. aeruginosa* clinical and environmental isolates were amplified and sequenced, allowing the identification of variant forms of these receptor genes.

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

Strain	*fpvA* type	*fpvB* gene (562 bp)	Source
PAO1	I	Positive	Stover *et al.*, 2000
ATCC 27853	IIb	Negative	Spencer *et al.*, 2003
7NSK2	IIa	Positive	De Chial *et al.*, 2003
59.2O	III	Positive	De Chial *et al.*, 2003
W15 Aug 16	III	Positive	Woluwe River water
W15 Dec 9	III	Positive	Woluwe River water
W15 Dec 10	III	Positive	Woluwe River water
W15 Aug 15	IIa	Positive	Woluwe River water
W15 Dec 11	IIb	Positive	Woluwe River water
W15 Dec 1	I	Positive	Woluwe River water
W15 Dec 6	I	Positive	Woluwe River water
W15 Aug 21	I	Positive	Woluwe River water
Br678	II	Positive	Burn wound (Pirnay *et al.*, 2005)

Table 1. List of strains used in this study

database for the purpose of amplification and sequencing of the ferripyoverdine 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 bacterial 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.

	Primer name	Sequences 5' → 3'
External primers for *fpvA* type I gene amplification	1A-PF	ATGCCAGCACCACACGGTC
	1A-PR	GCGAGTGCTGAACATCAGGT
Internal primers for sequencing the *fpvA* type I gene	Int1-AF1	CCTGATCCGCAAGAAACCT
	Int1-AR1	GGCCGCTGACGTCCAGTT
	Int1-BF1	GTGACCGATGACCTGAACC
	Int1-BR1	CGGACTCGCGAATGGTCGG
External primers for *fpvA* type IIa gene amplification	2A-PF	CAGCACTCCGCACACTCC
	2A-PR	GTCAAGTCGGAGGTGATGG
Internal primers for sequencing the *fpvA* type IIa and IIb genes	EFT-2A	TCCAGTTCGACATCGGCGC
	Int2-AF2	CAAGCGCAGCGGTACCGGC
	Int2-AR2	AACCGCGCGCCTTGATCTC
	Int2-BF2	CGACCGCGACCAGTTCGT
	Int2-BR2	TGCCGAA(G/C)(A/G/C)CTTTGTTGGT
	2A-int2AF	AATCAATCGGCCGCCATGC
External primers for *fpvA* type IIb gene amplification	ExtF-2A	GCGCAACACCCTGACG(C/T)TGC
	ExtR-2A	TTACCAGTTGTAGGTCAG(G/A)TCG
External primers *fpvA* type III gene amplification	EFT-IIIA	ATGTCCAGGCTTGGCCTGGC
	ERT-IIIA	GTAGTCGACCCAGGAGCG
	ExFN-IIIA	GAGCGCCCAGGCGGTCGC
Internal primers for sequencing the *fpvA* type III gene	Int3AF3	TGCGCAAGCGACCGACGG
	Int3AR3	GCCGGCCTTGGCAGTCAG
	Int3BF3	ACGCTTCGATCCAGAAAGGC
	Int3BR3	ACTCCTTGGCCACCGCCTTC
	Int3-BF3	
	FpVAint3F	AACCTGCTGCGTTACCAGG
	FpVA-3PR	AGCACGTTGCGCGGATCG
External primers for *fpvB* gene amplification	PA4168F	CCATCCAGGAACTGCAGAT
	PA4168R	GGATCAGAGCGATACTTCA
Internal primers for sequencing the *fpvB* gene	FpvbF1	AGGCAGCGTCGGCACCTG
	FpvbF2	AAACGTCGGCCTCGCTGC
	FpvbR1	CTGAAAGTGATCCTCGGCG
	FpvbR2	CTTGTACCAGTCCAGGCG
Degenerate primers for amplification and sequencing the *fpvA* type II gene*	FpVAF1	AGA CCG A (T/C) A CCC CGC TCA AG
	FpVAF2	(C/A)G(C/G)(C/G)(T/C)(G/A)(G/A)(T/C)(C/A)(C/A)(T/G)(G/A)(A/G)C(T/A)(T/C)C(A/G)(C/A)G
	FpVAR1	CTT GGT GTA CTT GA (T/C) GTC GTT (T/G)
	FpVAR2	G (T/A) G GGT (A/G)T A G(C/G)C (A/G) GC (C/A)T(G/A) (A/T)ACC

* Degenerate primer used to amplify and sequence *fpvA* gene in *P. aeruginosa* strain Br678.

Table 2. List of external and internal primers designed and used in this study.

Reagents	Volume (µL)
Water	774.0
Buffer	120.0
Q-solution	240.0
Primers (100 µM)	6.0 each
dNTP mix (10 mM)	24
Enzyme (*Taq* polymerase)	6.0
DNA	2

Table 3A. PCR mix for *fpvB* gene amplification using primers PA4168F and PA4168R (Ghysels *et al.*, 2004)

	Temperature (°C)	Time	
Initial denaturation	94	3-5 min	
Denaturation	94	30 s	
Annealing	55	30 s	35 cycles
Extension	72	2.30 min	
Final extension	72	10 min	

Table 3B. PCR cycling conditions

Reagents	Volume (µL)
Water	258
Buffer	40
Q-solution	80
$MgCl_2$	12
Primers (100 µM)	2 each
dNTP mix (10 mM)	8
Enzyme (*Taq* polymerase)	2
DNA	2

Table 3C. PCR mix for *fpvA* type I gene amplification using primers 1A-PF and 1A-PR

Reagents	Volume (µL)
Water	516
Buffer	80
Q-solution	160
$MgCl_2$	20
Primers (100 µM)	4 each
dNTP mix (10 mM)	16
Enzyme (Taq polymerase)	4
DNA	2

Table 3D. PCR mix for *fpvA* gene amplification using primers FpVAF1, FpVAF2, FpVAR1, and FpVAR2

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:

Strain	*fpvA* type	Source
W15 Aug 21	I	Woluwe River water
W15 Dec 1	I	Woluwe River water
W15 Dec 6	I	Woluwe River water
W15 Aug 15	IIa	Woluwe River water
W15 Dec 11	IIb	Woluwe River water
59.2O	III	*De Chial et al.*, 2003
W15 Aug 16	III	Woluwe River water
W15 Dec 9	III	Woluwe River water
W15 Dec 10	III	Woluwe River water

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

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:

- FpVBF1 (forward) from position (803-820) of aligned forward sequences;
- FpVBF2 (reverse) from position (833-850) of aligned forward sequences;
- FpVBR1 (forward) from position (1609-1628) of aligned reverse sequences; and
- FpVBR2 (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)
DNA preparation method. Sterile colonies of 12 bacterial samples (eight Woluwe River water strains that included *fpvA* types I-III plus four positive controls) were grown and templates were prepared for PCR as described above.

Strain	**Source**	***fpvB* (2.5 kb)** This study
(PAO1 (+)	Stover *et al.*, 2000	Positive
ATCC 27853(+)	Spencer *et al.*, 2003	Negative
7NSK2 (+)	De Chial *et al.*, 2003	Positive
59.2O (+)	De Chial *et al.*, 2003	Positive
W15 Aug 16	Woluwe	Positive
W15 Dec 9	Woluwe	Positive
W15 Dec 10	Woluwe	Positive
W15 Aug 15	Woluwe	Positive
W15 Dec 11	Woluwe	Positive
W15 Dec 1	Woluwe	Positive
W15 Dec 6	Woluwe	Positive
W15 Aug 21	Woluwe	Positive
Other Reference strains		***fpvA* type**
PA 14		I
10-15	Smith *et al.*, 2005	I
MSH	Smith *et al.*, 2005	II
1-60	Spencer *et al.*, 2003	II
2-164	Spencer *et al.*, 2003	II
ATCC013	Smith *et al.*, 2005	III
206-12	Smith *et al.*, 2005	III

Table 5. Additional information for strains used in this study.

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 *fpvA* gene of three *P. aeruginosa* strains as shown below:

Strain	*fpvA* type	Source
W15 Aug 21	I	Woluwe River water
W15 Dec 1	I	Woluwe River water
W15 Dec 6	I	Woluwe River water

Table 6. Three *P. aeruginosa* strains

The resulting sequences were aligned with the complete sequence of the *fpvA* gene of three reference strains (PAO1, PA14, and 10-15) as described above. The alignment was done for the *fpvA* sequences of three reference strains plus all resulting forward sequences (for three sequenced strains) and the *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 *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 *fpvA* gene.
***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 *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 and100 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 *fpvA* gene of *P. aeruginosa* strain W15 Aug 15 as shown below:

Strain	*fpvA* type	Source
W15 Aug 15	IIa	Woluwe River water

Table 7. *P. aeruginosa* strain W15 Aug 15

The resulting sequence was aligned with the sequence of the *fpvA* gene of five reference strains (7NSK2, ATCC 27853, MSH, 2-164, and 1-60) as described above. The alignment was done for the *fpvA* gene sequences of five reference strains plus resulting forward sequences (for 1 sequenced strain) and the *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 *fpvA* gene; the internal primers designed are shown below:

- EFT-2A (forward) from position (500-519) of aligned forward sequences;
- Int2AF2 (forward) from position (801-819) of aligned forward sequences;
- Int2AR2 (Reverse) from position (880-898) of aligned forward sequences;
- Int2BF2 (forward) from position (1537-1554) of aligned reverse sequences;
- Int2BR2 (Reverse) from position (1736-1754) of aligned reverse sequences;
- 2A-Int2AF (forward) from position (2344-2362**)** of aligned reverse sequences; and 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 (ExtF-2A and ExtR-2A) and only internal primers shown in bold above were used.

***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 *fpvA* type III gene amplification using primers EFT-II1A and 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 *fpvA* gene of three *P. aeruginosa* strains as shown below:

Strain	*fpvA* type	Source
W15 Aug 16	III	Woluwe River water
W15 Dec 10	III	Woluwe River water
W15 Dec 9	III	Woluwe River water

Table 8. Three *P. aeruginosa* strains.

The resulting sequences were aligned with the sequence of the *fpvA* gene of three reference strains (59.2O, ATCC 013, and 206-12) as described above. The alignment was done for the *fpvA* sequences of three reference strains plus all resulting forward sequences (for three sequenced strains) and the *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 *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.

Strain	**Source**	**Pvd siderotype** (Pirnay *et al.*, 2005)	***fpvB* (562 bp)** Primers (Ghysels *et al.*, 2004)	***fpvB* (2.5 kb)**
Br678	Burn	II	Positive (+)	NA, not analyzed

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

Strains	***fpv type***
PAO1, PA14, 10-15	I
7NKS2, ATCC27853, MSH, 1-60, 2-164	II
59.20, ATCC013, 206-12	III

Primers	**Position**	**Tm (°C)**
FpVAF1	836-855	67.4
FpVAF2	836-855	53.8
FpVAR1	2501-2522	60.5
FpVAR2	2501-2522	60.4

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 *fpvA* sequences of 11 *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.
***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 *fpvA* sequence of Br678 was 96% identical and 97% similar to *fpvA* type II of *P. aeruginosa* isolate 2-164 at nucleotide and amino acid levels, respectively.

4. Amplification of *fpvB* gene (2.5 kb) in *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 (PA4168F and PA4168R as above); these primers were used to sequence the external portions of the *fpvB* gene of strain Br678.
In addition, the two sets of internal primers previously designed for the sequencing of the internal portion of the *fpvB* gene were used to sequence the internal portion of the *fpvB* gene in this strain as shown below:

- FpVBF1 (forward) from position (803-820) of aligned forward sequences;
- FpVBF2 (Reverse) from position (833-850) of aligned forward sequences;
- FpVBR1 (forward) from position (1609-1628) of aligned reverse sequences; and
- 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. *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.

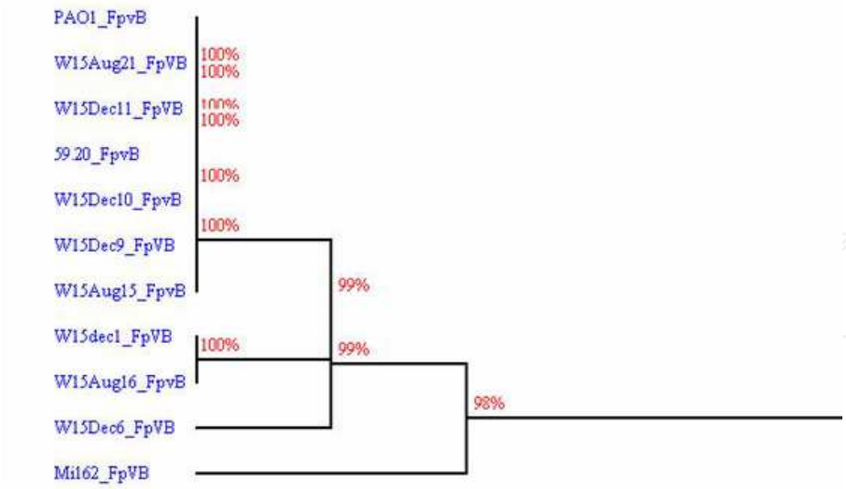
6. Results

The nucleotide sequences of both *FpvA* and *FpvB* determined in this study have been deposited in the GenBank database (Bodilis *et al.*, 2009).

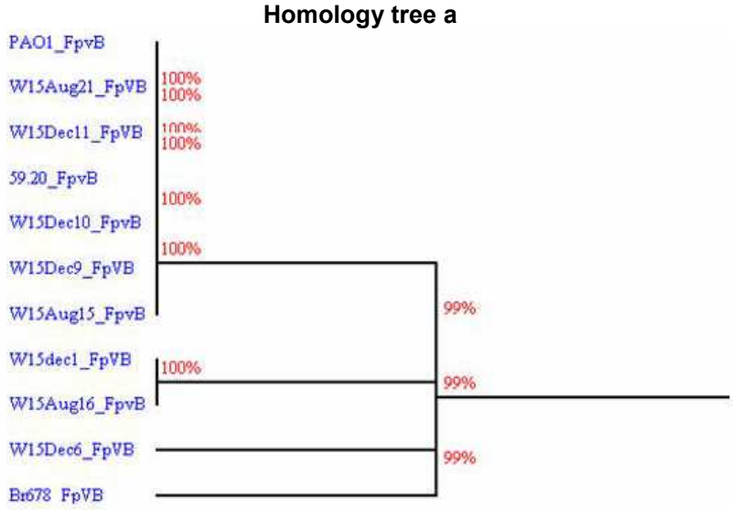
Primer name	*fpvA* type	*fpvA* (bp)	Primer name
1A-PF	I	~2500	PA4168F
1A-PR			PA4168R
Int1-AF1			FpvbF1
Int1-AR1			FpvbF2
Int1-BF1			FpvbR1
Int1-BR1			FpvbR2
2A-PF	II a	~2500	PA4168F
2A-PR			PA4168R
EFT-2A			FpvbF1
Int2-AF2			FpvbF2
Int2-AR2			FpvbR1
Int2-BF2			FpvbR2
Int2-BR2			
2A-int2AF			
ExtF-2A	II b	~2500	PA4168F
ExtR-2A			PA4168R
Int2-AF2			FpvbF1
Int2-AR2			FpvbF2
Int2-BF2			FpvbR1
Int2-BR2			FpvbR2
EFT -IIIA	III	~2500	PA4168F
ERT-IIIA	III		PA4168R
ExFIIIAN	III		FpvbF1
FpVA-3PR			FpvbF2
Int3AF3			FpvbR1
Int3BF3			FpvbR2
int3BF3			
Int3-BF3			
Int3BR3			
FpVAint3F			
FpVAF1	II	1500	PA4168F
FpVAF2			PA4168R
FpvAR1			FpvbF1
FpvAR2			FpvbF2

Table 11. External and internal primers used in this study.

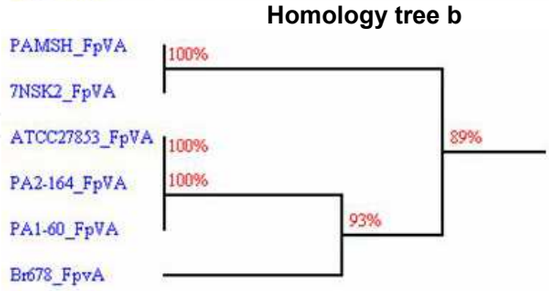
fpvB (bp) ~2500 bp for the primers used



Homology tree a



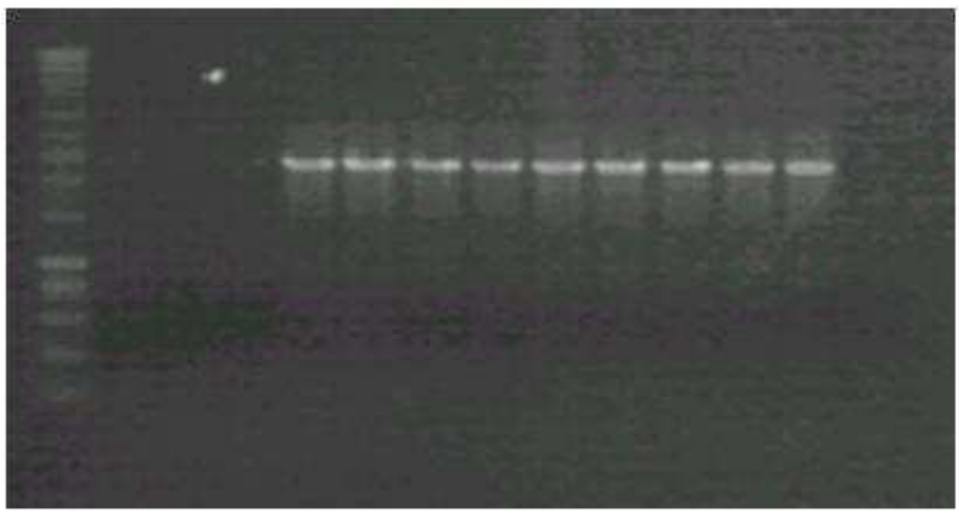
Homology tree b



Homology tree c

Fig. 1. Homology trees (a-c) show the percent relatedness of the *fpvA* and *fpvB* genes in *P. aeruginosa* strains *Br678* and *Mi162* (*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 (*fpvA* and *fpvB*) sequences for the individual *P. aeruginosa* test and reference strains.

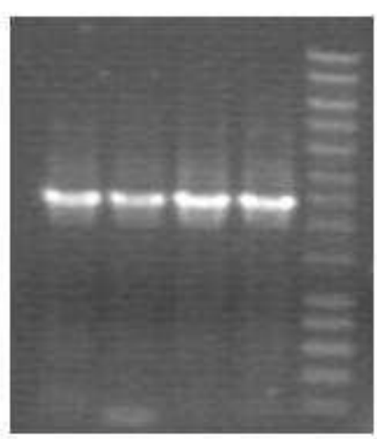
7. Gel images of *fpvA* and *fpvB* gene amplification in *P. aeruginosa* strains



L 1 2 3 4 5 6 7 8 9 10 11 12 13

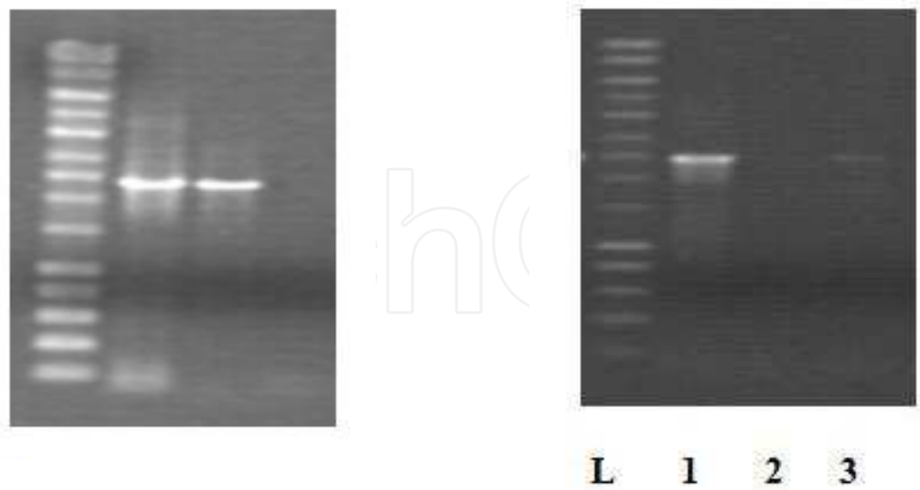
Fig. 2. Gel image of *fpvB* (2.5 kb) gene amplification in *P. aeruginosa* strains. Lanes 1-13: PAOI (positive control), ATCC 27853, 7NSK2, 59.20, W15 Aug 21,

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).



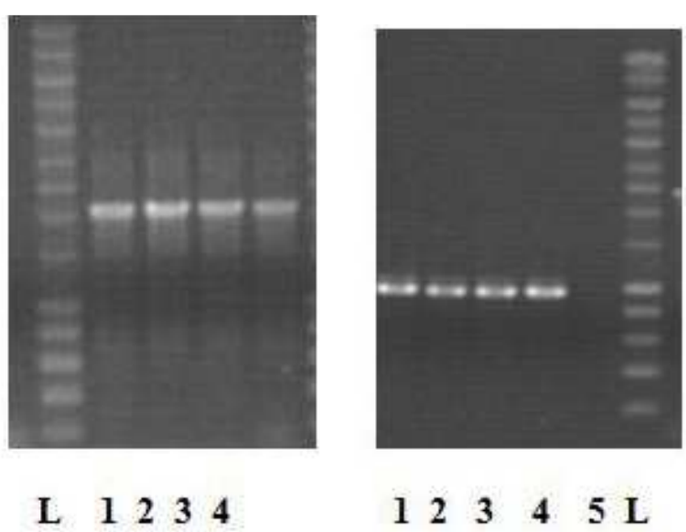
1 2 3 4 5 L

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).



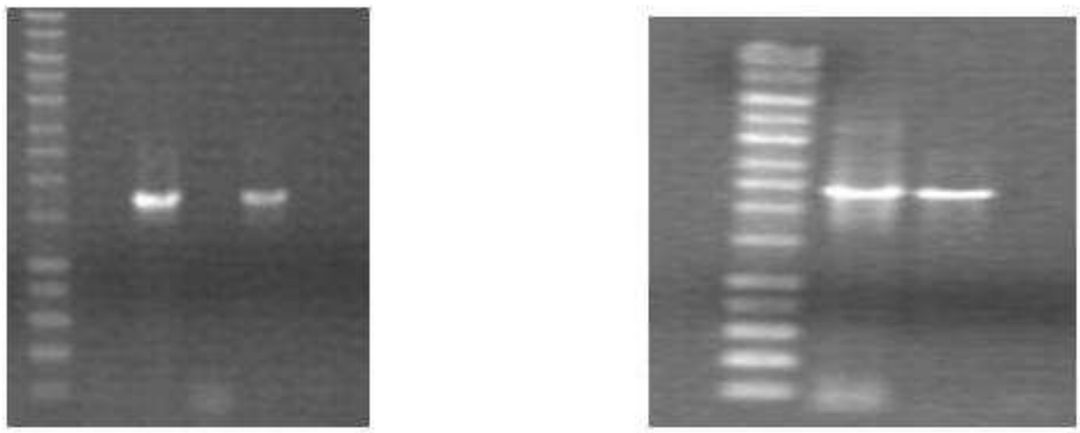
(a) (b)

Fig. 4. Gel image of *fpvA* (2.5 kb) gene amplification in *fpvA* type IIa *P. aeruginosa* strains (a). Lanes 1-3: 7NSK2 (positive control), W15 Aug 15, and negative control; L, 200-bp ladder. Gel image of *fpvA* (2.5 kb) gene amplification in *fpvA* type IIb *P. aeruginosa* strains (b). Lanes 1-3: ATCC 27853 (positive control), negative control, W15 Dec 11; L, 200-bp ladder).



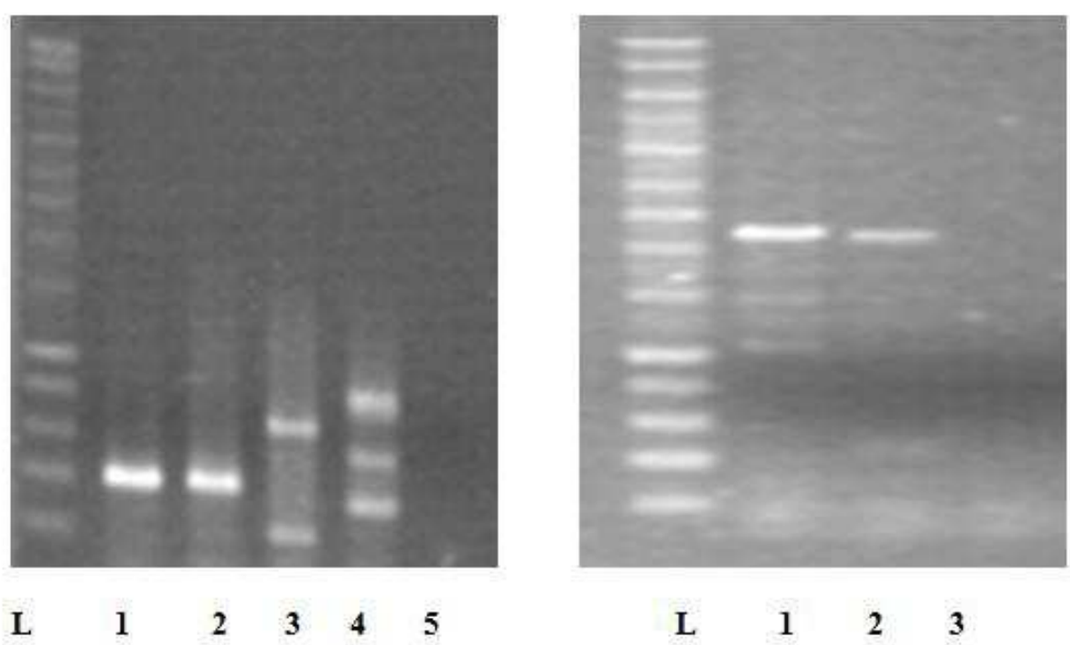
(a) (b)

Fig. 5. Gel images of *fpvA* (2.0 and 1.0 kb) gene amplification in *fpvA* type III *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.



(a) (b)

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.



(a) (b)

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)

	Strain	Pvd type	Source	*fpvA*: % identity (nucleotide level)	*fpvB*: % identity (nucleotide level)
1	59.2O	III	De Chial *et al.*, 2003		99% identical to *fpvB* of PAO1
2	W15 Aug 21	I	Woluwe	99% identical to *fpvA* of PAO1	99% identical to *fpvB* of PAO1
3	W15 Dec 1	I	Woluwe	99% identical to *fpvA* of PAO1	99% identical to *fpvB* of PAO1
4	W15 Dec 6	I	Woluwe	99% identical to *fpvA* of PAO1	99% identical to *fpvB* of PAO1
5	W15 Aug 15	11a	Woluwe	99% identical to *fpvA* of 7NSK2	99% identical to *fpvB* of PAO1
6	W15 Dec 11	11b	Woluwe	95% identical to *fpvA* of ATCC27853	99% identical to *fpvB* of PAO1
7	W15 Aug 16	III	Woluwe	100% identical to *fpvA* of ATCCO13	99% identical to *fpvB* of PAO1
8	W15 Dec 9	III	Woluwe	100% identical to *fpvA* of ATCCO13	99% identical to *fpvB* of PAO1
9	W15 Dec 10	III	Woluwe	100% identical to *fpvA* of ATCCO13	99% identical to *fpvB* of PAO1
10	Br678	II	Burn	97% identical to ferrichrome iron receptor PA7	99% identical to *fpvB* of PAO1
11	Mi162	II	Burn	99% identical to PA strain MSH type II pyoverdine region	98% identical to *fpvB* of PA14

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

	Strain	Pvd type	Source	*fpvA*: % similarity (amino acid level)	*fpvB*: % similarity (amino acid level)
1	59.2O	III	De Chial *et al.*, 2003		100% similar to ferric coprogen and ferric rhodoturolic acid of PA 2192
2	W15 Aug 21	I	Woluwe	96% similar to *fpvA* of UCBPP-PA14	100% similar to ferric coprogen and ferric rhodoturolic acid of PA 2192
3	W15 Dec 1	I	Woluwe	99% similar to *fpvA* of PAO1	99% similar to ferric coprogen and ferric rhodoturolic acid of PA UCBPP-PA14
4	W15 Dec 6	I	Woluwe	100% similar to *fpvA* of PAO1	99% similar to *fpvB* PAO1
5	W15 Aug 15	11a	Woluwe	99% similar to *fpvA* of PA C3719	99% similar to ferric coprogen and ferric rhodoturolic acid of PA 2192
6	W15 Dec 11	11b	Woluwe	99% similar to *fpvA* of PA MSH	99% similar to *fpvB* of PAO1
7	W15 Aug 16	III	Woluwe	100% similar to *fpvA* of PA206-12	99% similar to ferric coprogen and ferric rhodoturolic acid of PA UCBPP-PA14
8	W15 Dec 9	III	Woluwe	100% similar to *fpvA* of ATCCO13	99% similar to ferric coprogen and ferric rhodoturolic acid of PA 2192
9	W15 Dec 10	III	Woluwe	100% similar to *fpvA* of ATCCO13	99% similar to *fpvB* PAO1
10	Br678	II	Burn	98% similar to *fpvA* of PA C3719	99% similar to ferric coprogen and ferric rhodoturolic acid of PA 2192
11	Mi162	II	Burn	100% similar to *fpvA* II *Pseudomonas aeruginosa*	99% similar to *fpvB* second ferripyoverdine receptor of PA UCBPP-PA14

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

9. Discussion

Under iron limiting conditions, pyoverdine is produced by *Pseudomonas aeruginosa*, a human opportunistic pathogen, several studies track its occurrence as a noscomial pathogen indicating that antibiotic resistance is increasing in clinical isolates (**which may be true for the strains I worked with**). Pyoverdine is a metal chelating compound, *P. aeruginosa*, in the past has been studied to acquire plasmid (Mercer and Loutit, 1979), several transporting mechanisms have been extensively studied in this organism of which is the pyoverdine transport. Based on the different pyoverdine types, three siderovars which exists within the *P. aeruginosa* group have been detected by siderotyping, (Fuchs *et al.*, 2001; Meyer *et al.*, 1997; 2002); this technique, however, is limited. The existence of pyoverdine-negative isolates of *P. aeruginosa* (De Vos *et al.*, 2001) has prompted the need for accurate and enhanced genotyping procedures based on the determination of the gene sequence of ferripyoverdine receptor *fpvA* of *P. aeruginosa*. Cloning techniques have been used to characterize iron-regulated genes in *P. aeruginosa* (Poole *et al.*, 1993; Visca *et al.*, 1994; Cunliffe *et al.*, 1995, McMorran *et al.*, 1996; Ochsner *et al.*, 2002; Ochsner and Vasil, 1996), and several simple and inexpensive methods exist for the typing of *P. aeruginosa* isolates (AL-Samarrai *et al.*, 2000). Several studies have involved the use of PCR-based techniques in the identification and characterization of Gram-negative bacteria (Bej *et al.*, 1991; De Vos *et al.*, 1997; Fricker and Fricker, 1994; Mcintosh *et al.*, 1992; Laguerre *et al.*, 1994; Kasai *et al.*, 1998; Anzai *et al.*, 2000; Qin *et al.*, 2003) using PCR-specific primers designed for the amplification of genes in these organisms. PCR is not limited by pyoverdine production as is siderotyping and, as such, is considered reliable and less time-intensive than cloning. Since PCR-specific primers are designed to carry out amplification procedures, the problems with false positives may not likely arise, although this has not always been the case. *P. aeruginosa* secretes pyocyanin which has been documented to strip iron from transferrin (Cox, C. 1986), it also produces pyoverdine, which strips iron and contributes to the virulence of this organism, thus pyoverdine production is accompanied by virulence factor secretion (Meyer, 1996; Clarke *et al.*, 2001; Beare *et al.*, 2003). Pyoverdine growth stimulation assays have also been used to type *P. aeruginosa*, but situations have arisen whereby the observable growth had been stimulated by more than one pyoverdine (Pirnay *et al.*, 2002; Meyer, 1992; Meyer *et al.*, 1999; Stintzi *et al.*, 2000). The outcome of such assays may only be predictions, and, therefore, not of use for molecular diagnosis (Pirnay *et al*. 2005). The *fpvA* gene sequence has been proposed to be diverse (Thupvong *et al.*, 1999; Smith *et al.*, 2005); with such diversity, it may be difficult to perform PCR on such a gene without problems of non-specific amplification. However, in this study, single bands arising as a result of *fpvA* or *fpvB* gene amplification were either purified directly or excised and purified from the gel. Results of sequencing following a BLAST search against the NCBI database revealed that these sequences were approximately 95-100% identical and similar at both the nucleotide and amino acid levels to those of reference strains used in this study. Homology trees showing percent relatedness of the *fpvA* and *fpvB* genes in *P. aeruginosa* test and reference strains were also constructed using the DNA manager software following an alignment of all nucleotide sequences for the individual test and reference strains.

The purpose of this study was to use PCR for the determination of about 2.5 kb gene sequence of the ferripyoverdine receptor genes (*fpvA* types *I*, *IIa*, *IIb*, and *III*, and *fpvB*) in *P. aeruginosa* clinical and environmental strains, and this goal was achieved using a series of external and internal primers designed for both amplification and sequencing. This study

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.

10. 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 amongst 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.

11. Citation

J. Osayande : Use of Polymerase Chain Reaction for the Determination of about 2.5 kb fpvA and fpvB Gene Sequences in *Pseudomonas aeruginosa* Strains. *The Internet Journal of Microbiology.* 2009 Volume 7 Number 2

12. Acknowledgement

I am grateful to Professor P. Cornelis and the Vrije Universiteit Brussels (VUB) for the provision of Academic Scholarship.

13. References

[1] Al-Samarrai, T.H, Zhang, N., Lamont, I.L., Martin, Lois, Kolbe, J., Wilsher, M., Morris, A.J., and Schmid, J. 2000. Simple and inexpensive but highly discriminating method for computer-assisted DNA fingerprinting of *Pseudomonas aeruginosa*. J. Clin. Microbiol. 38(12): 4445-4452.

[2] Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H., and Oyaizu, H. 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. 50: 1563-1589.

[3] Beare, P.A., For, R.J., Martin, L.W., and Lamont I.L. 2003. Siderophore-mediated cell signaling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. Mol. Microbiol. 47 (1): 195-207

[4] Bej, A.K., McCarty, S.C., and Atlas, R.M. 1991. Detection of coliform bacteria and Eschericia coli by multiplex polymerase chain reaction: Comparison with defined substrate and plating methods for water quality monitoring. Appl. Environ. Microbiol. 57(8): 2429-2432.

[5] Bodilis, J., Ghysels, B., Osayande, J., Matthijs, S., Pirnay, J.P., Denayer, S., De Vos, D., and Cornelis, P. 2009. Distribution and evolution of ferripyoverdine. April 21 [Epup ahead of print].

[6] Clarke, T.E., Tari, L.W., and Vogel, H.J. 2001. Structural biology of bacterial iron uptake systems. Curr. Top. Med. Chem. 1:7-30.

[7] Cornelis, P., Hohnadel, D., and Meyer, J.M. 1989. Evidence for different pyoverdine-mediated iron uptake systems among *Pseudomonas aeruginosa* strains. Infect. Immun. 57:3491-3497.

[8] Cox, C.D. 1986. Role of Pyocyanin in the acquisition of iron from transferrin. Infect. Immun. 52(1): 263-270.

[9] Cunliffe, H.E., Merriman, T.R., and Lamont, I.L. 1995. Cloning and characterisation of pvdS, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. J. Bacteriol. 177: 2744-2750.

[10] De Chial, M., Ghysels, B., Beatson, S., Geoffroy, V., Meyer, J. M., Pattery, T., Baysse, C., Chablain, P., Parsons, Y.N., Winstanley, C., Cordwell, S., and Cornelis, P. 2003. Identification of type II and type III pyoverdine receptors from *Pseudomonas aeruginosa*. Microbiology 149: 821-831.

[11] De Vos, D., Lim, J. A., Pirnay, J.P., Struelens, M., Vandenvelde, C., Duinslaeger, L., Vanderkelen, A., and Cornelis, P. 1997. Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR Based on two outer membrane lipoprotein Genes, OprI and OprL.J. Clin. Microbiol. 35(6): 1295-1299.

[12] De Vos, D., De Chial, M., Cochez, C., Jansen, S., Tummler, B., Meyer, J.M., and Cornelis, P. 2001. Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine –negative mutations. Arch. Microbiol. 175:384-388.

[13] Fricker, E.J., and Fricker, C.R. 1994. Application of the polymerase chain reaction to the identification of Escherichia coli and coliforms in water. Lett. Appl. Microbiol. 19: 44-46.

[14] Fuchs, R., Schafer, M., Geoffroy V., and Meyer, J.M. 2001. Siderotyping – A powerful tool for the characterization of pyoverdines. Curr. Top. Med. Chem. 1:31-57.

[15] Gensberg, K., Hughes, K., and Smith, A.W. 1992. Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 138:2381-2387.

[16] Ghysels, B., Dieu, B.T., Beatson, S.A., Pirnay, J.P., Ochsner, U.A., Vasil, M.L., and Cornelis, P. 2004. FpvB, an alternative type I ferripyoverdine receptor of *Pseudomonas aeruginosa*. Microbiology 150: 1671-1680.

[17] Guerinot, M.L. 1994. Microbial iron transport. Annu. Rev. Microbiol. 48: 743-72.
[18] Karry Mullis Nobel Lecture, December 8, 1993.
[19] Kasai, H., Watanabe, K., Gasteiger, E., Bairoch, A., Isono, K., Yamamoto, S., and Harayama, S. 1998. Construction of the gyrB database for the identification and classification of bacteria. Genome Inform. 9:13-21.
[20] Laguerre, G., Gois-Rigottier, L., and Lemanceau, P. 1994. Fluorescent Pseudomonas species categorized by using polymerase chain reaction (PCR)/ restriction fragment analysis of 16S rDNA. Mol. Ecol., 3: 479-487.
[21] Mcintosh, I., Govan, J.R.W., and Brock, D.J.H. 1992. Detection of *Pseudomonas aeruginosa* in sputum from cystic fibrosis patients by the polymerase chain reaction. Mol. Cell. Probes 6: 299-304.
[22] McMorran, B.J., Merriman, M.E., Rombel, I.T., and Lamont, I.L. 1996. Characterization of the pvdE gene which is required for pyoverdine synthesis in *Pseudomonas aeruginosa*. Gene 176: 55-59.
[23] Mercer, A.A and Loutit , J.S. 1979. Transformation and transfection of *Pseudomonas aeruginosa*: Effects of Metal Ions. J. Bacteriol. 140 (1) 37-42.
[24] Meyer, J.M. 1992. Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. J. Gen. Microbiol. 138:951-958.
[25] Meyer, J.M., Geoffroy, V.A., Baida, N., Gardan, L., Izard, D., Lemanceau, P., Achouk, W., and Palleroni, N. 2002. Siderophore typing, a powerful tool for the identification of fluorescent and non-fluorescent pseudomonads. Appl. Environ. Microbiol. 68: 2745-2753.
[26] Meyer, J.M., Neely, A., Stintzi, A., Georges, C., and Holder, I.A. 1996. Pyoverdine is essential for virulence of Pseudomonas aeruginosa. Infect. Immun. 64: 518-523.
[27] Meyer, J.M., Stintzi, A., and Poole, K. 1999. The ferripyoverdine receptor FpvA of *Pseudomonas aeruginosa* PAO1 recognizes the ferripyoverdines of P. aeruginosa and P. fluorescens ATCC 13525. FEMS Microbiol. Lett. 170: 145-150.
[28] Pirnay, J.P., De Vos, D., Cochez, C., Bilcoq, F., Vanderkelen, A., Zizi, M., Ghysels, B., and Cornelis, P. 2002. *Pseudomonas aeruginosa* displays an epidemic population structure. Environ. Microbiol. 4: 898-911.
[29] Pirnay, J.P., Matthijs, S., Colak, H., Chablain, P., Bilocq, F., Van Eldere, J., De Vos, D., Zizi, M., Triest L., and Cornelis, P. 2005. Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. Environ. Microbiol. 7(7): 969-980.
[30] Poole, K., Neshat, S., and Heinrichs, D. 1991. Pyoverdine-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high –molecular –mass outer membrane protein. FEMS Microbiol. Lett. 78: 1-6.
[31] Poole, K., Neshat, S., Krebes, K., and Heinrichs, D.E. 1993. Cloning and nucleotide sequence analysis of the ferripyoverdine receptor fpvA of *Pseudomonas aeruginosa*. J. Bacteriol. 175: 4597-4604.
[32] Qin, X., Emerson, J., Stapp, J., Stapp, L., Abe, P., and Burns, J.L. 2003. Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting Gram-negative Bacilli from patients with cystic fibrosis. J. Clin. Microbiol. 41: 4312-4317.
[33] Quadri, L.E.N. 2000. Assembly of aryl-capped siderophores by modular peptide synthetases and polyketide synthases. Mol. Microbiol. 37(1): 1-12.

[34] Smith, E.E., Sims, E.H., Spencer, D.H., Kaul, R., and Olson, M.V. 2005. Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. J. Bacteriol. 187(6): 2138-2147.

[35] Spencer, D. H., Kas, A., Smith, E. E., Raymond, C. K., Sims, E. H., Hastings, M., Burns, J. L., Kaul, R., and Olson, M. V. 2003. Whole-genome sequence variation among multiple isolates of *Pseudomonas aeruginosa*. J. Bacteriol. 185, 1316–1325.

[36] Stinzi, A., Barnes, C., Xu, J., and Raymond, K.N. 2000. Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm. Proc. Natl. Acad. Sci. USA, 97: 10691-10696.

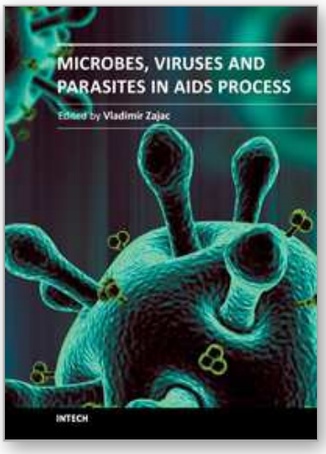
[37] Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino E, Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E., Lory, S., Olson, M.V. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406(6799):959-64.

[38] Thupvong, T., Wiideman, A., Dunn, D., Oreschalk, K., Jankowicz, B., Doering, J., and Castignetti, D. 1999. Sequence heterogeneity of the ferripyoverdine uptake (fpvA), but not the ferric uptake regultaor (fur), genes among strains of the fluorescent pseudomonads *Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas fluorescens and Pseudomonas putida*. Biometals 12: 265-274.

[39] Todar, K. 2004.The Good, the Bad and the Deadly. Science Magazine, 304: Pg. 1421

[40] Tummler, B., and Cornelis, P. 2005. Pyoverdine Receptor: a case of Darwinian selection in *Pseudomonas aeruginosa*. J. Bacteriol. 187(10): 3289-3292.

[41] Visca, P., Ciervo, A., and Orsi, N. 1994. Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdine biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. 176(4): 1128-1140.



Microbes, Viruses and Parasites in AIDS Process

Edited by Prof. VladimÃr Zajac

ISBN 978-953-307-601-0
Hard cover, 390 pages
Publisher InTech
Published online 19, October, 2011
Published in print edition October, 2011

The main goal in compiling this book was to highlight the situation in Africa in terms of AIDS and opportunistic diseases.Â Several chapters reveal great poverty, an apocalyptic situation in many parts of Africa.Â 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.Â 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.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Julie Osaretin Osayande (2011). Use of Polymerase Chain Reaction for the Determination of About 2.5 kb fpvA and fpvB Gene Sequences in Pseudomonas aeruginosa Strains, Microbes, Viruses and Parasites in AIDS Process, Prof. VladimÃr Zajac (Ed.), ISBN: 978-953-307-601-0, InTech, Available from: http://www.intechopen.com/books/microbes-viruses-and-parasites-in-aids-process/use-of-polymerase-chain-reaction-for-the-determination-of-about-2-5-kb-fpva-and-fpvb-gene-sequences-

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821