IDENTIFICATION AND CHARACTERIZATION OF THE GENES ENCODING FOR SECONDARY SIDEROPHORE IN PSEUDOMONAS FLUORESCENS SBW25

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Abtract
To be able to survive and grow, Pseudomonas fluorescens and many micro organisms need iron as a source of nutrition. In order to satisfy their requirement for iron under conditions of iron limitation, fluorescent pseudomonads secrete a high-affinity siderophore pyoverdine to scavenge Fe (III) in the extracellular environment and to shuttle it into the cell. Beside siderophore pyoverdine, there are also other kinds of siderophore produced by fluorescent pseudomonads, which are called "secondary siderophores''. We are interested in these secondary siderophores because they were thought to play a more diverse role than only uptake of iron. In addition to iron, some secondary siderophores also chelate other ions such as: Zn(II), Cu(II), Mo(VI), Ni(II) and may promote their uptake in fluorescent Pseudomonas (Cornelis and Matthijs, 2002). It has also been shown that secondary siderophores have anti-microbial properties (Matthijs et al, 2007).
Based on Tn5 mutagenesis, we identified and characterized 2 genes involved in secondary siderophore synthesis. Both genes encode for a putative non-ribosomal peptide synthetase (NRPS). This type of enzyme generates a peptide chain of some siderophores.Based on the obtained sequences and with several bioinformatics tools, we predicted a putative secondary siderophore synthesis operon that consists of 12 open reading frames (ORFs). BLAST comparison revealed a function for each ORF.
In order to further investigate the predicted gene function constructs were made to knock out 6 of the 12 identified genes. To complement the mutants a genomic library of Pseudomonas fluorescens SBW25 was also constructed.

Key words: *Pseudomonas fluorescens* SBW25, siderophore, siderophore pyoverdine, secondary siderophores**,** nonribosomal peptide synthetase
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INTRODUCTION

Pseudomonas Fluorescens are gram negative bacteria, can motile with polar flagella, living under aerobic condition and adopt chemoorganotrophic metabolism. To be able to survive and grow, those micro-organisms need iron as a source of nutrition.

Iron exists in nature in two forms, under aerobic conditions, it forms Fe^{3+} which is insoluble and under anaerobic conditions, it forms Fe^{2+}, which is soluble. However, majority of iron are in insoluble forms which are unusable for microbial. So it is a big problem. So what is solution for this? The answer is the ability to produce Siderophore-which are high iron chelators.

Pseudomonas fluorescens produced siderophore and it was secreted out the cell into the environment. Then, it will bind to iron and forms a complex called ferri-siderophore. This complex will be transported into the cell via a specific outer membrane receptor and the iron will be reduced to Fe^{2+}. Many previous studies showed that the first siderophore is pyoverdine. However, beside pyoverdine, those microorganism can still be able to produce other siderophores- which are called '' Secondary siderophores''. And we are interested in these secondary siderophore because they were thought playing more important role than pyoverdine. They are not only involved in the iron uptake, but also in other essential metal ions, such as: Cu, Ni, Zn…

So the goal of this study is to identify the genes, which are responsible to these secondary siderophores production.

MATERIALS & METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are shown in Table 1.

Table 1 *Strains and plasmids used in this study*

Strains		Descriptions	Source of reference
Pseudomonas fluorescens			
	SBW25	Wild-type strain isolated from phyllosphere of sugar beet plant	Rainey and Bailey, 1996
	pvdL	Deletion mutant SBW25::*pvdL*	Moon C., unpublished
	6H9	Pf::*pvdL*::mini-Tn5*phoA3*, Gm^R	This study
	10B3	Pf::*pvdL*::mini-Tn5*phoA3*, Gm^R	This study
	10G1	Pf::*pvdL*::mini-Tn5*phoA3,* Gm^R	This study
	15F3	Pf::*pvdL*::mini-Tn5*phoA3,* Gm^R	This study
Phytopathogen			
Pythium debaryanum		Sugar beet isolate	P. Lepoivre, Gembloux
E.coli			
	SM10 λ *pir*	*Thi-1 thr leu tonA lacY SupE recA*:: RP4-2 tc::Mu; λ pir; Km^R	Herrero *et al.* (1990)
	S17-1 λ *pir*	*thi pro hsdR recA*: :RP4-2-Tc^R: :Mu Km^R: :Tn7 (λ*pir*), Sm^R	Simon *et al*. 1993
	DH5α	*end* A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *gyr*A *rel*A1Δ(*lac*IZA-*arg*F) U169 *deo*R (Φ80d*Lac*Δ (*lacZ*) M15), $Na1^R$	Woodcock *et al*. 1989
	VCS 257	Gigapack III Gold Packaging Extract (Stratagene)	
	CM404	Bearing plasmid pRK2013	Boyer and Roulland-Dussoix, 1996
λ phage		Gigapack III Gold Packaging Extract (Stratagene)	
Plasmids	pUK21	Cloning vector, lac Zα, Km^R	Vieira and Messing, 1991
	pUKORF2	pUK21 carrying a 1340 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF2, Km^R	This study
	pUKORF3	pUK21 carrying a 2330 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF3, Km^R	This study
	pUKORF4	pUK21 carrying a 2000 nt *Hin*dIII-*Bam*HI insert with a deletion in ORF4, Km^R	This study
	pUKORF5	pUK21 carrying a 2000 nt *Bam*HI-*Eco*RI insert with a deletion in ORF5, Km^R	This study
	pUKORF10	pUK21 carrying a 1900 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF10, Km^R	This study
	pUKORF11	pUK21 carrying a 1800 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF11, Km^R	This study
	pME3087	Suicide vector, ColE1 replicon, RK2-Mob, Tc^R	Voisard *et al.*, 1994

pME6.2	pME3087 carrying a 2330 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF3, Tc^R	This study
pME21.2	pME3087 carrying a 1900 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF10, Tc^R	This study
pME30.2	pME3087 carrying a 1800 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF11, Tc^R	This study
pME4.1	pME3087 carrying a 2000 nt *Hin*dIII-*Bam*HI insert with a deletion in ORF4, Tc^R	This study
pME17.2	pME3087 carrying a 2000 nt *Bam*HI-*Eco*RI insert with a deletion in ORF5, Tc^R	This study
pMER15	pME3087 carrying a 1340 nt *Hin*dIII-*Eco*RI insert with a deletion in ORF2, Tc^R	This study
pUT mini- Tn*pho*A3	Mini-Tn*5phoA3*, Gm^R	Dr. Pattery, VUB
pRK2013	Mob(RP4), Km^R, used as helper plasmid in mobilisation	Figursky *et al*., 1982
pRG930^{cmR} (cosmid)	cosmid cloning vector, pVS1 origin of replication 16.1 kb Sm^R/Sp^R	Van den Eede *et al*., 1992 Matthijs *et. al*., 2004

Construction of mini-Tn5phoA3 mutants. A schematic overview of the methodology used in this study is illustrated in Figure 2.1.

Mutagenesis was done by biparental mating of *P. fluorescens pvdL* (Pf::*pvdL*) with donor strain *E. coli* SM10 λ *pir* containing the suicide plasmid pUT and transposon mini-Tn*5phoA3*. A filter-plate mating procedure was performed by mixing mid-log phase culture of *E. coli* SM10 λ *pir*, the host of pUT-mini-Tn*5phoA3*, with Pf::*pvdL* in a 1:1 ratio. Following incubation of the mating mix on LB for overnight at 28°C, transposon-mutagenised *P. fluorescens* recipients were selected on CAA medium supplemented with 100 µg/ml *Gm* and 25 µg/ml *Cm* + 100 µM Fe. A bank of 3000 transposon–mutagenized Pf::*pvdL::* mini-Tn*5phoA3* was established to screen for the loss of capacity to produce a secondary siderophore.

After two rounds of confirmation using the CAS assay, mutants were selected for further characterization.

Self- ligation. Purified chromosome DNA of mutant strains was digested by the restriction enzyme SalI or PstI, then self-ligated in 100µl with enzyme T4 ligase (Fermentas) overnight at 16°C.

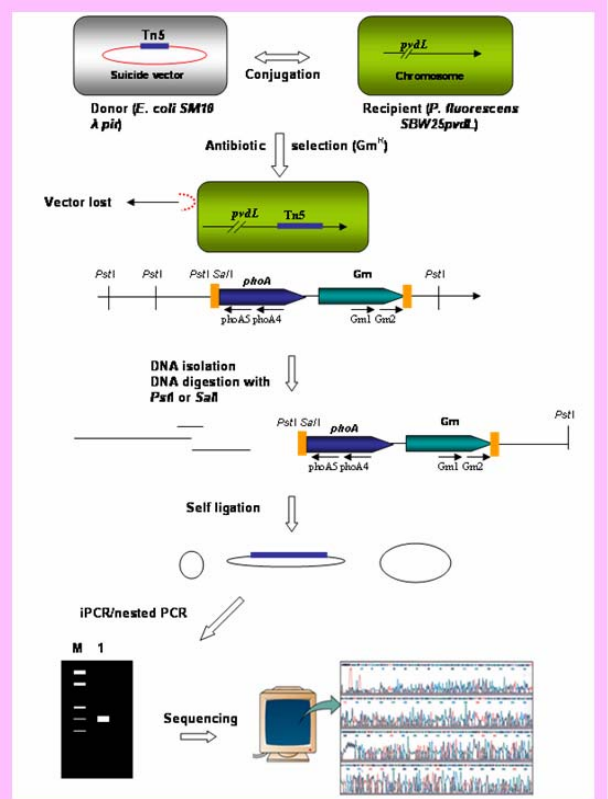

Figure 2.1 *A schematic overview of the mini-Tn5PhoA3 mediated mutant methodology*

Inverted PCR (iPCR) and nested PCR. IPCR was performed by using the two primers PhoA5(5'GCGGCAGTCTGATCACCCGTTA3') and Gm1(5'TGGACCAGTTGCGTGAGCGCATA3') to amplify the regions immediately flanking the transposon.

Nested-PCR was carried out after the first amplification with two different primers *PhoA4* (5'GCACCGCCGGGTGCAGTAATTAT 3') and *Gm2* (5'TGTCAACTGGGTTCGTGCCTTC3'). PCR program consisted with 30 cycles included 30s denaturation at 94°C, 30s annealing at 55°C and 4 min elongation at 72°C for each cycle.

Sequencing determination. The amplified fragments were then purified using a Qiagen PCR purification kit and sequenced directly at the VIB sequencing facility (http://www.vib.be).

Database searching. DNA sequence analysis and comparisons with sequences contained in the Genbank and EMBL databases were done with the DNAMAN software. The obtained sequences were also compared with sequences in the databank of the *Pseudomonas* genome (http://www.pseudomonas.com) and the *P. fluorescens* SBW25 sequencing project (http://www.sanger.ac.uk/Projects/P_fluorescens/) using the BLASTX algorithm.

Phenotypic characterization of the mini-Tn*5phoA3* mutants. The phenotypic characterization of double mutants included a CAS siderophore detection assay, a growth assay and an *in-vitro* antagonism assay against *P. debaryanum*.

Construction of gene deletion mutants. The to be inactivated gene was deleted from the coding region by insertion of a plasmid containing two DNA fragments derived from the flanking regions of the to be deleted gene by homologous recombination.

In the first step, both flanking regions located in up and down stream region of deleted gene were amplified by PCR (signed as A and B). These two PCR fragments were cloned in pUK21. The PCR primers were designed in a way that provides an *Xba*I site between the joined fragments as well as two different restriction sites at the two ends.

Subsequently, the plasmid was sequenced to select candidates with no amplification error or deletions in the primers.

The PCR products (A and B) were then recloned into pME3087 using the same restriction enzymes.

E. coli S17-1 λ *pir* competent cells were then transformed with this construct and a conjugation with *P. fluorescens* SBW25::*pvdL* was carried out. Colonies of transconjugants were selected on CAA medium supplied with 10 µg/ml tetracycline and 25 µg/ml chloramphenicol. The antibiotic resistant colonies were used to screen for mutants by PCR reaction.

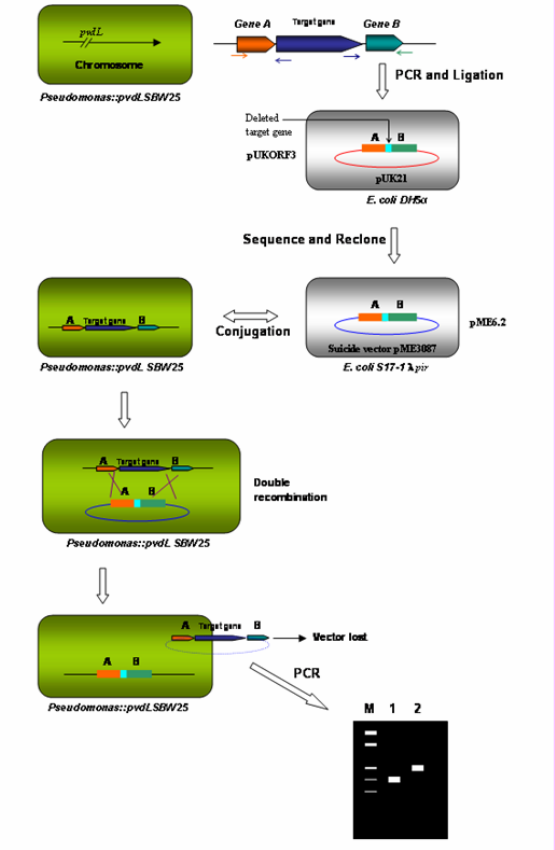

Figure 2.2 *A schematic overview of the deletion mediated mutant methodology*

Transformation. Standard procedures for DNA transformation were performed as described by Sambrook *et al.*. 200 µl of suspension was plated on selective medium plates containing LB+50 µg/ml Km. The control tube was maintained using the same procedure without

addition of ligation mixture and spread on the same medium. The resistant clones should grow on selective medium, whereas no clones should be seen on the control plate.

Conjugation. A filter-plate mating procedure was performed by mixing mid-log phase culture of *E.coli* S17λ *pir*, the host of suicide plasmid pME3087, pME6.2, pME21.2, pME30.2, pME4.1, pME17.2 or pMER15, with *P. fluorescens pvdL* in a 1:1 ratio. Following incubation of the mating mix on LB for 4h at 28°C, mutagenized *P. fluorescens* recipients were selected for on CAA medium supplemented with 10 μg/ml Tc and 25 μg/ml Cm.

λ phage packaging

Packaging of the ligation mixture, containing cosmid pRG930^{CmR} packaged with partially digested genomic DNA fragments into bacteriophage λ was done by using Gigapack IV Gold packaging extract (Stratagene) following the manufacturer instructions with *E. coli* VCS257 as host strain. The positive clones were selected on LB medium supplied with 50 μg/ml Cm and 50 μg/ml Sp.

RESULTS

Transposon mutagenesis was carried out on the *pvdL* mutants to identify the genetic loci that are involved in secondary siderophore synthesis. A total of 3000 colonies were screened for mutants that lost secondary siderophore production using a CAS assay. Four CAS-negative mutants were obtained after screening, which are named 6H9, 10B3, 10G1, and 15F3.

The siderophore production of the wild type, the pyoverdine-negative and double pyoverdine-secondary siderophore negative mutants was estimated by the CAA-CAS assay. *P. fluorescens* SBW25 wild type produced a large halo around the inoculated spot, due to the production of pyoverdine and 2nd siderophore on CAA-CAS agar.

The pyoverdine negative *pvdL* mutant produced a smaller halo due to the production of the second siderophore. All the 6H9, 10B3, 10G1 and 15F3 double mutants failed to produce a halo around the colony, indicating that they lost the capacity to produce secondary siderophores.

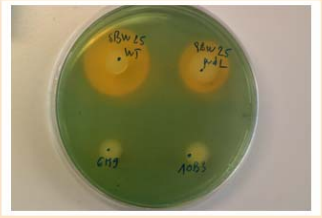

Figure 3.4 *The result of the CAS assay. Three measurements were taken for each mutant.P.fluorescens SBW25 wild type and pvdL pyoverdine negative mutant produced a smaller halo round, but 6H9 and 10B3 double mutants did not show a halo around the colony, indicating that they lost the siderophore production.*

Total genomic DNA of 4 double mutants 6H9, 10B3, 10G1, and 15F3, respectively was isolated for iPCR and nested PCR. iPCR was performed to amplify the regions immediately flanking the mini-Tn*5phoA3* by using specific primers (*PhoA5* and *Gm1*) designed for mini-Tn*5phoA3*. Subsequently, nested PCR was carried out to re-amplify iPCR fragments by using nested primers *PhoA4* and *Gm2*.

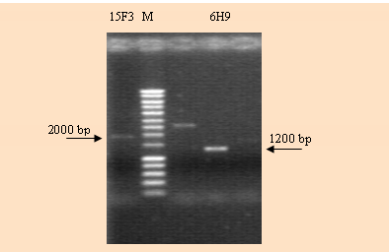

Figure 3.2. *0,8% agarose electrophoresis analysis of the products of iPCR reactions carried out with genomic DNA of pvdL:Tn5PhoA3 mutants 6H9, 10B3, 10G1, 15F3. Lane 2: DNA Smartladder maker (eurogentec). Lane 1, 4: iPCR products from reactions carried out with genomic DNA pvdL:Tn5PhoA3 mutants 15F3, 6H9 respectively.*

IPCR products appear as only one band of approximately 1200 bp and 2000 bp for 6H9 and 15F3 mutants, respectively. However, no iPCR product was obtained for either 10G1 or 10B3 mutant. The fact that the lengths of the iPCR fragments for 6H9 and 15F3 were different suggests that the mini-Tn*5PhoA3* transposon was inserted at different positions in the genome.

To predict the function of the genes which transposon were inserted, the sequences of the flanking regions of the mini-Tn*5phoA3* were used as template to search for other similar gene/protein sequences using Blastn/BlastX programs on website: http://www.ncbi.nlm.nih.gov/blast/ or http://v2.pseudomonas.com/blast.jsp. Briefly, the similarity search is shown in the Table 2. The results suggested that genes were interrupted by mini-Tn*phoA3* in *P. fluorescens* SBW25 genome is similar to non-ribosomal peptide synthetase (ppsD) of *P. putida* KT2440.

Table 2 *Sequence analysis of putative NRPS genes from 6H9 and 15F3 mutants*

Mutants	Similarity
6H9	Similar to siderophore **non-ribosomal peptide synthetase** (*PpsD*) of *P. putida* KT2440. Expected = 1.0E-41; identities = 44.52%
15F3	Similar to siderophore **non-ribosomal peptide synthetase domain protein, putative** (*PpsD*) of *P. putida* KT2440. Expected = 3.0E-4; identities = 26.04%

Based on the sequence results of putative NRPSs in 6H9 and 15F3 mutants, a nucleotide fragment about 47 kb from nucleotide positioned 3522954 to 3569465 of SBW25 genome has been annotated for ORFs by using FGENESB program on the website http://www.softberry.com.

Twelve ORFs were predicted (from ORF1 to ORF12) and they are lying side by side in the same orientation. The position of the mini-Tn*5* inserted into 6H9 and 15F3 double mutant strains is located at ORF 7 and ORF 8, respectively.

To predict the putative function of each ORF, similarity search of ORF sequences against known function sequences in Genebank was done by using the BLAST program at the website http://v2.pseudomonas.com/doBlastAlignment.do. The starting and ending base, molecular mass (kDa), pI value, the predicted location and the similarity for each ORF are shown in Table 3.

Table 3 *Products of the 12 ORFs, their predicted function and localization (Cyto: cytoplasm; OM: outer membrane; IM: inner membrane)*

ORF	Start base	Ending base	AA	kDa	pI	Location	Similarity
1	1012	2427	471	54,3	6,76	Cyto	Similar to diamino -butyrate--2-oxoglutarate transaminase of *Pseudomonas syringae* with 61.4% identity and expected value: 2.0E-165
2	2545	4578	677	71,8	5,48	OM	Similar to putative TonB dependent receptor in *P. aeruginosa* PAO1 with 30% identity and expected value: 5.0E-61
3	4862	5839	325	37,7	5,19	Cyto	Similar to putative SyrP protein in *P. putida* KT2440 with 57,84% identity , expected value: 2.0E-102
4	5848	9219	1123	127	5,53	Cyto	Similar to acylCoA -synthetase in *Pseudomonas syringae pv. syringae str.* B728a with 34,78% identity, expected value: 1.0E-144
5	9237	10283	348	40,4	5,4	Cyto	Similar to putative SyrP protein in *P. putida* KT2440 with 46,5% identity, expected value: 3.0E-77
6	10318	13329	1003	111,4	5,13	Cyto	Similar to probable non-ribosomal peptide synthetase in *P. aeruginosa* PAO1 with 36% identity , expected value: 1.0E-104
7	13376	27286	4636	525,8	5,07	cyto	Similar to putative non ribosomal peptide synthetase (*PpsD*) in *Pseudomonas putida* KT2440 with 37.39% identity, expected value: 3.0E-

8	27341	37405	3354	382,7	5,04	cyto	160 Similar to putative non-ribosomal peptide synthetase (*PpsD*) in *P. putida* KT2440 with 38.98% identity, expect value: 3.0E-177
9	37402	41148	1248	143,6	11,61	Cyto	Similar to putative non-ribosomal peptide synthetase domain protein, in *P. putida* KT2440 with 34% identity, expect value: 1.0E-99
10	41149	42780	543	63,2	5,84	IM	Pyoverdine ABC export system in *Pseudomonas fluorescens* Pf-5 with 37% identity and expect value: 3.0E-79
11	42771	43454	227	20,6	10,97	Cyto	Similar to putative FeS chemotaxis transducer in *P. aeruginosa* PAO1 with 50% identity, expect value: 7.0
12	43654	45357	567	66,5	5,96	IM	Similar to ABC transporter, ATP-binding protein in *P. syringae phaseolicola* 1448A with 39% identity, expect value: 5.0E-97

The organization of 12 putative ORFs is shown in Fig 4.2.

Two putative NRPS modules of 6H9 and 15F3 were identified by using "Domain search program". The findings showed 4 putative modules in ORF7 and 2 modules in ORF8. Schematic overview of 6H9 and 15F3 NRPS modules is shown in Figure 4.1.

By using "NRPS predictor" program, we predicted that four amino acids, X-Asp-Ser-Ser, are activated by the NRPS in ORF7 and that two amino acids, Asp-X, are activated in ORF8. Amino acid X means that no match was found. These results suggested that there are at least six amino acids present in the *P. fluorescens* SBW25 secondary siderophore.

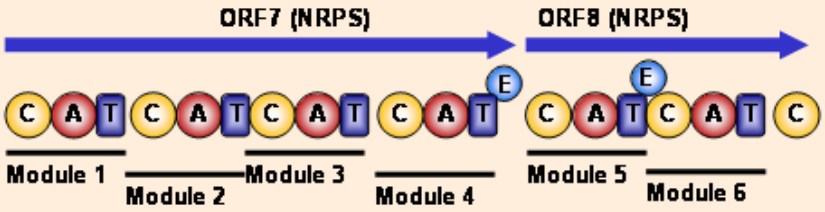

Figure 4.1 Putative *NRPS modules of secondary siderophore peptide chain synthesis in* P. fluorescens *SBW25.*

The function of each gene could be predicted by similarity alignment with closely related genes the functions of which are known. To increase the confidence of the prediction, 6 genes were selected, which were considered to be interesting. These were the putative receptor gene (Orf 2), a gene encoding a transporter (Orf 10), SyrP regulators (Orf 3 and Orf 5), a gene encoding a putative acyl-CoA synthetase (Orf 4), and a gene encoding a putative iron-sulfur cluster protein (Orf 11). These genes were independently knocked-out by deletion mutagenesis to see the effect on secondary siderophore production.

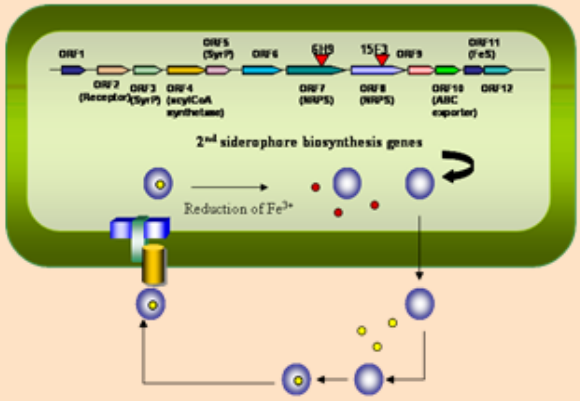

Figure 4.2 *Putative secondary siderophore sythesis operon in* Pseudomonas fluorecens *SBW25*

For this, 12 constructs to make the deletion mutants were made. After the construction of the mutants, further characterization of the mutants is

required to further determine their function by doing CAS assays, and measuring the growth parameters.

As an example, it will be interesting for the receptor mutant to verify whether it is involved in the uptake of the secondary siderophore. For this a small assay can be carried out whereby the mutant is inoculated on CAA + a non-usable iron chelator and CAA + iron chelator + secondary siderophore-Fe. If the receptor mutant cannot grow on these media, it indicates that the mutant cannot take up the siderophore-iron complex, thereby confirming the role of the receptor. If the mutant can grow on the media amended with the secondary siderophore-Fe complex, it would reveal that the receptor has not been inactivated because it is still able to take up the siderophore-iron complex. Alternatively, there may exist another redundant uptake system as already observed for the uptake of ferripyoverdine where next to the FpvA receptor, a second receptor, FpvB can mediate the uptake of the ferrisiderophore (Ghysels *et al*., 2004). A more deep analysis would consist out of uptake experiments with ^{59}Fe-labelled iron-siderophore complexes.

The regulator mutants can likewise be tested on CAS medium. If the siderophore production is reduced, compared to that of Pf::*pvdL*, it indicates that the genes code for an activator. In contrast, if the iron uptake capacity of the mutants are increased, the genes probably encode a repressor.

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