Pyocin S2 (Sa) Kills *Pseudomonas aeruginosa* Strains via the FpvA Type I Ferripyoverdine Receptor[▽][†]

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Soluble (S-type) pyocins are *Pseudomonas aeruginosa* bacteriocins that kill nonimmune *P. aeruginosa* strains via a specific receptor. The genes coding for pyocin Sa (consisting of a killing protein and an immunity protein) were cloned and expressed in *Escherichia coli*. Sequence analysis revealed that Sa is identical to pyocin S2. Seventy-nine strains of *P. aeruginosa* were tested for their sensitivity to pyocins S1, S2, and S3, and their ferripyoverdine receptors were typed by multiplex PCR. No strain was found to be sensitive to both S2 and S3, suggesting that the receptors for these two pyocins cannot coexist in one strain. As expected, all S3-sensitive strains had the type II ferripyoverdine receptor *fpvA* gene, confirming our previous reports. S1 killed strains irrespective of the type of ferripyoverdine receptor they produced. All S2-sensitive strains had the type I *fpvA* gene, and the inactivation of type I *fpvA* in an S2-sensitive strain conferred resistance to the S2 pyocin. Accordingly, complementation with type I *fpvA* in *trans* restored sensitivity to S2. Some S2-resistant type I *fpvA*-positive strains were detected, the majority (all but five) of which had the S1-S2 immunity gene. Comparison of type I *fpvA* sequences from immunity gene-negative S2-sensitive and S2-resistant strains revealed only a valine-to-isoleucine substitution at position 46 of type I FpvA. However, both type I *fpvA* genes conferred the capacity for type I pyoverdine utilization and sensitivity to S2. When these two type I *fpvA* genes were introduced into strain 7NSK2 carrying mutations in type II *fpvA* (encoding the type II pyoverdine receptor) and *fpvB* (encoding the alternative type I receptor), growth in the presence of type I pyoverdine was observed and the strain became sensitive to S2. We also found that type I pyoverdine could signal type II pyoverdine production via the type I FpvA receptor in 7NSK2.

Pseudomonas aeruginosa is a ubiquitous gram-negative gammaproteobacterium able to cause severe nosocomial infections and lung infections in cystic fibrosis patients (21). *P. aeruginosa* is also notorious for its high level of resistance to several antibiotics (17). Like other aerobes, *P. aeruginosa* produces siderophores to fulfill its needs for iron (9, 29, 37). The major siderophore of *P. aeruginosa* is the fluorescent pyoverdine, which has a conserved chromophore and a variable peptide chain (29, 37). Both the chromophore and the peptide chain are synthesized by nonribosomal peptide synthetases (29, 37). Three different pyoverdine types in *P. aeruginosa* can be distinguished, each having a specific receptor that coevolves with the nonribosomal peptide synthetase genes (8, 10, 23, 34, 35). Pyocins are bacteriocins produced by *P. aeruginosa* that kill other strains from the same species (24, 26). *P. aeruginosa* pyocins can be subdivided into insoluble (particular phage-like) R and F pyocins and soluble S pyocins, and only the S pyocins are protease and heat sensitive (24). Soluble pyocins have an N-terminal receptor binding domain, a translocation domain, and a C-terminal killing domain (24). The majority of S-type pyocins (AP41, S1, S2, and S3) cause cell death by DNA breakdown due to an endonuclease C-terminal domain (24),

while pyocin S4 is predicted to have tRNase activity and S5 is predicted to have pore-forming activity (24, 26). Pyocin-producing strains are protected from their own toxin by an immunity protein encoded by an adjacent gene (24). It is known that iron limitation greatly increases the killing activity of some S pyocins, including S2, S3, and Sa (11, 25, 33). Ohkawa et al. (25) first reported that mutants resistant to S2 fail to produce an iron-repressed outer membrane protein but did not establish the link with the ferripyoverdine receptor. Smith et al. (33) suggested that the pyocin Sa receptor could be the ferripyoverdine receptor, although they observed that a receptor-negative mutant, although resistant to Sa, still had some considerable residual pyoverdine utilization capacity, an observation that was not understood at that time but was later explained by the discovery of a second alternative type I ferripyoverdine receptor, FpvB (13). Later on, it was found that the receptor for type II ferripyoverdine is used by S3 to kill sensitive *P. aeruginosa* strains (1, 10). In this work, we show that pyocin Sa is in fact identical to pyocin S2 and that type I FpvA (the major type I ferripyoverdine receptor) is the receptor for this pyocin. The results presented here also suggest mechanisms of resistance to soluble pyocins other than the production of immunity proteins.

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MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. Strains were grown at 37°C in rich Luria broth (LB) medium (Life Technologies) or iron-poor Casamino Acids (CAA) medium (Difco Laboratories) in a New Brunswick Innova 4000 shaker at 200 rpm. The antibiotics spectinomycin, gentamicin, chloramphenicol, and ampicillin were used. The con-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
P. aeruginosa		
O:9	Wild-type pyocin Sa-sensitive indicator strain	33
O:9*fpvA*⁻	*fpvA* mutant of O:9	This work
W15Dec1	Wild-type pyocin S2-sensitive strain	27
W15Dec1*fpvA*⁻	*fpvA* mutant of W15Dec1	This work
W15Dec30	Wild-type pyocin S2-resistant strain	This work
W15Apr21	Wild-type pyocin S2-resistant strain	This work
W15Apr38.4	Wild-type pyocin S2-resistant strain	This work
PAO29P	Wild-type pyocin S2-resistant strain	J. P. Pirnay
J1003	Wild-type pyocin Sa-producing strain	14
7NSK2*fpvA*⁻*fpvB*⁻	Pyoverdine receptor-negative mutant; Gmʳ Tcʳ	13
E. coli		
DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15 recA hsdR17 recA1 endA1 gyrA96 thi-1 relA1)*	16
GJ23	JC2692(pGJ28)(R64*drd*11); Kmʳ Smʳ Tcʳ	36
S17.1 (λ pir)	Tpr Smr *recA thi pro hsdRM*⁺ RP4::2-Tc::Mu::Km Tn7 λ pir	32
Plasmids		
pBR325	ColE1 vector; Ampʳ Cmʳ Tcʳ	4
pBR325::*fpvAI*::*gm*	pBR325 with a 1,200-bp BamHI-EcoRI type I *fpvA* fragment disrupted with a Gm cassette; Cmʳ Ampʳ Gmʳ	This work
pBBR1*mcs*	Wide-host-range cloning vector; Cmʳ	20
pRG930-*cat*	Derivative of cosmid vector pRG930 with Cmʳ cassette; wide host range	22
pPYS3-3	Pyocin S3 gene-containing plasmid	11
pYMSS11	Pyocin S1 gene-containing plasmid	31
pYMPS1	Pyocin S2 gene-containing plasmid	31
pPVR2	pAK1900 derivative carrying *fpvA* on a 4.6-kb SphI fragment	28

[a] Gmʳ, Tcʳ, Kmʳ, Smʳ, Ampʳ, and Cmʳ indicate gentamicin, tetracycline, kanamycin, spectinomycin, ampicillin, and chloramphenicol resistance cassettes, respectively.

centrations used in different media for *Escherichia coli* and *P. aeruginosa* are mentioned below.

Sequence determination and analysis. Sequencing was done at the VIB Genetic Service Facility (University of Antwerp, Antwerp, Belgium), and sequences were compared with entries in the *P. aeruginosa* database (http://v2.pseudomonas .com).

Multiplex PCR for the identification of type I, II, and III *fpvA* genes and *fpvB*. The different *fpvA* genes were identified by multiplex PCR as described previously by De Chial et al. (10). Primers for the *fpvB* gene were included (13).

IEF and CAS overlay. An isoelectric focusing (IEF) and Chrome Azurol S (CAS) assay of partially purified pyoverdines from different *P. aeruginosa* isolates was performed as described by Koedam et al. (19) in order to differentiate type I, II, and III pyoverdines (23).

Isolation of pyoverdines. Pyoverdines for IEF and CAS analysis were partially purified from 4-ml cultures grown for 48 h (19). Type I pyoverdine for growth stimulation experiments was purified as described previously (23). The concentration of pyoverdine in a solution was estimated by measuring the absorbance at 400 nm ($\varepsilon = 2 \times 10^4$ M⁻¹ cm⁻¹) (23).

Pyoverdine growth stimulation assays. Growth stimulation by type I pyoverdine on CAA with 0.5 mg of ethylenediaminedihydroxyphenylacetic acid (EDDHA) ml⁻¹ was done by plating 5×10^6 cells of a *P. aeruginosa* culture per ml onto the agar surface. A filter disk impregnated with 5 μl of a 10 mM type I pyoverdine solution was deposited on top of the bacterial cell layer. Plates were incubated at 37°C, and growth was recorded after 24 and 48 h. Plates were photographed with a Fuji digital camera (Finepix S1 Pro).

Construction of a genomic library of *P. aeruginosa* J1003 and screening of S-type-pyocin-producing clones. Total DNA of *P. aeruginosa* J1003 was isolated using the blood and cell culture DNA midi kit (QIAGEN). Partially Sau3A-digested genome fragments were cloned into the BamHI site of pRG930-*cat* (22). Ligated DNA was packaged using the Gigapack III Gold-4 kit (Stratagene). About 2,200 clones were selected on LB agar plates containing 50 μg of spectinomycin ml⁻¹. To screen for S-type-pyocin-producing clones, the genomic library was replicated on LB agar plates containing 50 μg of spectinomycin ml⁻¹ and grown for 12 h. Cells were killed by chloroform vapors over a period of 20 min, and plates were kept open for another 20 min to eliminate all traces of chloroform. An overlay of soft LB agar (5 g of agar liter⁻¹) containing 5×10^6

cells of the pyocin Sa-sensitive indicator strain *P. aeruginosa* O:9 ml⁻¹ was used to screen for pyocin-producing clones. After incubation for 12 h, a clearance zone was seen. To distinguish S-type-pyocin-producing clones from others (those producing R- and F-type pyocins), a secondary screening of pyocin-producing clones was carried out with the addition of 100 μg of proteinase K ml⁻¹ to the agar overlay. Wild-type strain J1003 and *E. coli* DH5α carrying the empty pRG930-*cat* vector were used as control strains.

Subcloning of S-type-pyocin-expressing fragments from cosmid 2H12. Self-ligated PstI-digested cosmid 2H12 generated a subclone containing a ~10-kb insert and still producing an S-type pyocin. The 10-kb fragment was gel purified with a kit from QIAGEN, cut with HindIII, and subsequently ligated into the PstI-HindIII site of pBBR1*mcs*, yielding an approximately 4.5-kb fragment that was still able to express the pyocin. This fragment was further analyzed by sequencing.

Generation of Δ*fpvA* deletion mutants by allelic exchange. A 1,200-bp *fpvA* fragment was amplified using wild-type *P. aeruginosa* PAO1 genomic DNA and primers FpvABamHI (5′-GTG GGA TCC CAG TGC GAT CAA GGG CAA-3′) and FpvASalI (5′-GTG GTC GAC GCA GGT TCC AGT AGC TCT-3′). The PCR was performed using *Taq* polymerase (QIAGEN) with the following parameters: 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C for 30 cycles. The PCR product purified by using a QIAGEN kit was ligated into BamHI-SalI-restricted pBluescript KS(+) (Stratagene). The constructed plasmid was digested at a unique EcoRI site and ligated with a Gm cassette. This Gm cassette was obtained by PCR with transposon miniTn*5phoA3* (pGV4692) (10) as a template and primers GmFw1 (5′-GTG GAA TTC AAC GGA TGA AGG CAC GAA-3′) and GmRv1 (5′-GTG CTT AAG CGA TCT CGG CTT GAA CGA-3′). The PCR was carried out using *Taq* polymerase and the following parameters: 30 s at 94°C, 30 s at 51°C, and 1 min at 51°C for 30 cycles, followed by a terminal extension step for 10 min at 72°C. The construct was checked by sequencing.

The disrupted *fpvA* gene was then transferred into BamHI-SalI-digested pBR325 (Stratagene), competent *E. coli* DH5α cells were transformed with the plasmid, and chloramphenicol- and gentamicin-resistant clones were selected. Additional screening was performed by digestion. The selected clones were transferred into competent *E. coli* GJ23 cells for mobilization of the disrupted *fpvA* gene into S2-sensitive acceptor *P. aeruginosa* strain W15Dec1 by conjugation. Recombinants were selected on CAA agar plates containing 100 μg of

gentamicin ml^{-1} and 50 µg of spectinomycin ml^{-1} to counterselect for *E. coli*. Double recombinants were selected as those clones growing on CAA plates with 100 µg of gentamicin ml^{-1} and not in the presence of 300 µg of chloramphenicol ml^{-1}. Candidates were confirmed by PCR with primers FpvABamHI and FpvASalI, which resulted in the amplification of a 1,900-bp fragment from the mutants.

Construction of the type I *fpvA*(V46) and *fpvA*(I46) expression vectors. For the expression of type I FpvA carrying valine at position 46 [FpvA(V46)] in *trans*, a 3,956-bp PstI-ApaI fragment of pPVR2 was ligated into pBBR1*mcs*. Type I FpvA(I46) was generated by PCR using genomic DNA of *P. aeruginosa* PAO29p as the template and primers FpvAPstI (5′-GTG CTG CAG CGA CCA TCA TCG AGA TCA-3′) and FpvAXbaI (5′-GTG TCT AGA GGT AGC CGA GAA GCA GAA-3′). The PCR was performed using ProofStart polymerase in combination with *Taq* polymerase (according to the QIAGEN protocol) and the following parameters: 30 cycles of 10 s at 94°C, 1 min at 51°C, and 3 min at 68°C, with a final extension of 10 min at 68°C. The purified PCR product was then ligated into the XbaI-PstI site of pBBR1*mcs*. Constructs were confirmed by sequencing.

Complementation and heterologous expression of type I *fpvA*. Biparental matings with *E. coli* S17.1 λ pir bearing plasmid pBBR1*mcs*::*fpvA* (expressing FpvA with V46 or I46) were performed for the complementation of *P. aeruginosa* strain W15Dec1 carrying a mutation in *fpvA* (mutant strain W15Dec1*fpvA*$^-$) or for the heterologous expression of the ferripyoverdine receptor in strain 7NSK2 carrying mutations in type II *fpvA* and *fpvB* (mutant strain 7NSK2*fpvA*$^-$*fpvB*$^-$). The complemented mutants were selected on LB supplemented with 300 µg of chloramphenicol ml^{-1}.

SDS-PAGE analysis of outer membrane proteins. Outer membrane proteins from bacteria grown under iron-limiting conditions (in CAA medium) were prepared by following the differential Sarkosyl solubilization method (7, 12). Cells were obtained from 250-ml cultures after 48 h of growth by centrifugation at 4°C for 10 min at 7,000 rpm in a Sorvall RC5B SLA rotor. The pellet was resuspended in 4 ml of GTE (50 mM glucose, 25 mM Tris-HCl [pH 8.0], and 10 mM EDTA containing 4 mg of lysozyme per ml). After 30 min of incubation on ice, 20 ml of PMSF-Sarkosyl solution (2 mM phenylmethylsulfonyl fluoride, 2% *N*-lauroylsarkosine) was added. Next, the mixture was sonicated for 15 min or until it reached homogeneity. The total outer membranes were pelleted by ultracentrifugation at 28,000 rpm for 2 h at 4°C in a Beckman TL-100 centrifuge equipped with a Ti 1003 rotor. Finally, the pellet was resuspended in 1 ml of 25 mM Tris-HCl, pH 7.5. The protein content was determined by using the QuantiPro bicinchoninic acid assay kit (Sigma), and proteins were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE).

Pyocin sensitivity assays: spot method and CFU counting. To check the sensitivities of *P. aeruginosa* strains to pyocins S1, S2, and S3, 10 µl of pyocin lysate was spotted onto a bacterial cell layer containing 5×10^6 cells ml^{-1}. Pyocins were partially purified from *E. coli* DH5α bearing plasmid pYMSS11, pYMPS1, or pPYS3-3 expressing pyocin S1, S2, or S3, respectively (11, 31).

Alternatively, bacterial cells (5×10^6 cells ml^{-1}) were incubated at 37°C for 30 min with different amounts of pyocin S3. One hundred microliters of a 5×10^3 dilution was plated in triplicate onto CAA plates, and surviving colonies were counted after growth at 37°C for 24 h.

Screening for S-type pyocin production by *P. aeruginosa* isolates. To determine the type of S pyocin produced by different *P. aeruginosa* isolates, products from PCRs with the following primers specific for the pyocin types were assessed: S1S2imm-Fw (5′-CACAAGGGAGGGAAGTGA-3′) and S1S2imm-Rv (5′-GCCTTAAAGCCAGGAA-3′) for pyocins S1 and S2 and pycS3RB-fw (5′-CG TATCACGAGACAGGCA-3′) and pycS3RB-rv (5′-TGCCGCTTCTTCCGCT TT-3′) for pyocin S3. Because pyocins S1 and S2 share the same immunity protein, primers corresponding to the region encoding this protein were chosen. The PCR was done with *Taq* polymerase and the following conditions: 30 cycles of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C.

RESULTS

Molecular cloning of pyocin Sa. A genomic library of the Sa pyocin-producing strain J1003 was made in the wide-host-range cosmid vector pRG930-Cm (22). The recombinant *E. coli* clones were screened for their pyocin killing activity by using the Sa-sensitive *P. aeruginosa* O:9 strain as the indicator. In order to differentiate killing by R and F pyocins from killing by S pyocins, replicas were made with or without proteinase K in the top agar containing the sensitive strain. Out of 2,200
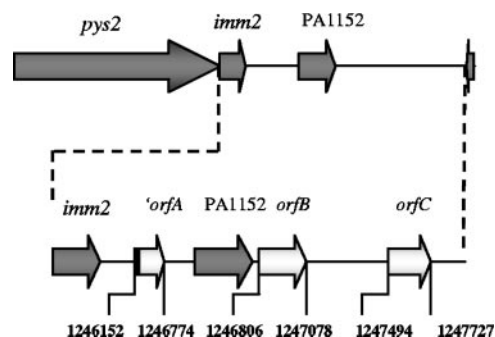

FIG. 1. *pys*2-PA1153 locus. The open reading frames in dark gray are those annotated in the PAO1 genome (http://www.pseudomonas .com), while those in white are predicted based on a BLASTX analysis. The first one (*orfA*) corresponds to part of a conserved hypothetical protein gene of the Tn*3* family of transposons. The two other open reading frames (*orfB* and *orfC*) encode a putative colicin immunity protein and a hypothetical protein, respectively. The region between *imm*2 and PA1153 is shown below in more detail. The genes are not to scale, but the numbers represent the nucleotide coordinates in the *P. aeruginosa* PAO genome.

colonies, 38 displayed killing activity, 15 of which produced a pyocin that was proteinase K sensitive. Ten clones with the highest level of protease-sensitive killing activity were selected, and their cosmids were extracted and restricted with SalI or PstI. Their restriction profiles were very similar, and two clones were selected for further work. After subcloning, a 4,589-bp PstI-HindIII fragment that conferred full killing activity was obtained. Sequencing of this fragment revealed two open reading frames corresponding to the pyocin killer protein and the immunity protein. Surprisingly, these open reading frames were 100% identical to the sequences annotated as *pys*2 (PA1150) and *imm*2 (PA1151), encoding pyocin S2 and the immunity protein, respectively, of *P. aeruginosa* PAO1 (Fig. 1). The fragment also contained the genes PA1152 (encoding a hypothetical protein) and PA1153 (carrying the COG3617 prophage antirepressor motif), as annotated in the *P. aeruginosa* PAO1 genome (http://www.pseudomonas.com). Via a BLASTX analysis, we found an incomplete reading frame (′*orfA*) corresponding to part of the *ofn44* gene (Q4W1R3_ PSEAE) from the IncP-6 plasmid Rsm149 (15) in the intergenic region between the *imm*2 gene and the PA1152 gene, which codes for a conserved hypothetical protein corresponding to the Tn*3* family of transposons (Fig. 1). Two other reading frames were found downstream of the PA1152 gene, one (*orfB*) encoding a putative colicin immunity protein (Q66CV8_YERPS) and the other (*orfC*) encoding a hypothetical protein (Q3KAI8_PSEPF). Based on these results, we can conclude that pyocin Sa is pyocin S2, since no other cosmid with protease-sensitive pyocin activity was found. Therefore, from now on this pyocin will be designated pyocin S2.

Determination of the specificity of pyocins S1, S2, and S3. Seventy-nine genotypically different *P. aeruginosa* strains from a previously molecularly typed collection (27) were screened for their sensitivities to three different S pyocins: S1, S2, and S3 (Table 2; see also Table S1 in the supplemental material). In parallel, the same isolates were subjected to a multiplex PCR analysis to type the ferripyoverdine receptor genes (type I, IIa, IIb, and III *fpvA* and *fpvB*) (13). No strain was sensitive to both

TABLE 2. Phenotypes of sensitivity or resistance to pyocins S1, S2, and S3 of 79 *P. aeruginosa* isolates[a]

Pyocin and phenotype	No. of isolates with:									No. of nontypeable isolates[c]
	Type I *fpvA*	Type I *fpvA* and S1-S2 *imm*	Type I *fpvA* and V→I mutation	Type II *fpvA*	Type II *fpvA* and S1-S2 *imm*	Type II *fpvA* and S3 *imm*	Type III *fpvA*	Type III *fpvA* and S1-S2 *imm*	
Pyocin S1										
Sensitive	14			9			6		
Resistant	18	9		20	9		9	4	3
Pyocin S2										
Sensitive	**17**								
Resistant	**15**	**9**[b]	**6**[b]	29	9		15		3
Pyocin S3										
Sensitive				**26**					
Resistant	32			**3**		**3**	15		3
Total	32	9	10	29	9	3	15	4	3

[a] In addition to the sensitivity testing, all strains were analyzed for the presence of type I, II, and III *fpvA* and *fpvB* by multiplex PCR (13). The presence of the S1-S2 and S3 immunity (*imm*) genes was checked by PCR as well. V→I indicates the presence of an isoleucine in place of a valine at position 46 of type I FpvA. Boldface indicates strains having *fpvAI* and *fpvAII* and sensitive to pyocins S2 and S3, respectively.

[b] Four strains with type I *fpvA* have both the S1-S2 immunity gene and the V→I mutation at residue 46.

[c] One strain could be identified as a type II pyoverdine producer by IEF but could not be typed by multiplex PCR.

S2 and S3 pyocins, indicating that the respective receptors for these two pyocins are not expressed in the same host (Table 2; see also Table S1 in the supplemental material). All 26 S3-sensitive strains had the type II pyoverdine *fpvA* gene (type IIa or IIb *fpvA*), confirming our previous results (1, 10). Ten strains were sensitive to S1 and S2 at the same time, eight were sensitive to S1 and S3, and S1 killed strains independently of the type of FpvA they produced (Table 2). All S2-sensitive strains (17 in total) had the type I ferripyoverdine *fpvA* gene. Intriguingly, 14 strains that were clearly shown by multiplex PCR to have the type I ferripyoverdine receptor gene were nevertheless resistant to S2 (Table 2). Nine of these S2-resistant strains carrying type I *fpvA* were shown to have the gene for immunity to S1 and S2, which is the same for the two pyocins (24, 31). The remaining five S2-resistant strains carrying type I *fpvA* were not positive by PCR for the S1 and S2 immunity gene, meaning that their resistance had another origin. Three of these strains were positive for type I *fpvA* but did not produce pyoverdine, which may result in reduced expression of the receptor (37). Interestingly, the well-studied and genome-sequenced type I pyoverdine producer PA14 had no S1-S2 immunity gene but was resistant to S2 (results not shown).

Sequence analysis of the type I *fpvA* receptor from S2-resistant strains. Although our screening suggested that type I FpvA could be the receptor recognized by S2, we were intrigued by the existence of some S2-resistant strains having the gene for this receptor and most likely producing it, since pyoverdine production is dependent on the interaction of pyoverdine with the receptor (37). The full type I *fpvA* genes from *P. aeruginosa* strains O:9 (S2 sensitive) and PAO29P (S2 resistant) were amplified and sequenced. A comparison of the two sequences revealed that there were only two nonsynonymous substitutions, resulting in amino acid changes at positions 24 (I→M) and 46 (V→I). Since position 24 is in the signal peptide, it is unlikely to have an impact on the function of the receptor, but residue 46 is in the N-terminal region of FpvA, which interacts with the FpvR antisigma factor (2, 30, 38). PA14, which is also resistant to S2 pyocin and does not have the immunity gene, has the same V→I substitution at position 46.

Effect of inactivation of type I *fpvA* on pyocin S2 sensitivity. The type I *fpvA* gene from the S2-sensitive strain W15Dec1 was inactivated by allelic replacement, resulting in the insertion of a Gm cassette. Likewise, the type I *fpvA* gene in O:9 was inactivated, but work with this strain was stopped because of difficulties in maintaining plasmids in this particular host (results not shown). The W15Dec1 type I *fpvA* mutant was unable to grow in the presence of EDDHA but could grow when type I pyoverdine was added to the growth medium because of the presence of the *fpvB* gene (13). The W15Dec1 type I *fpvA* mutant also became fully resistant to pyocin S2 (Fig. 2A). Complementation with both type I *fpvA*(*V46*) and type I *fpvA*(*I46*) in *trans* restored the sensitivity to pyocin S2, suggesting that the substitution is not involved in the resistance to pyocin S2 (Fig. 2A). The production of the two forms of type I FpvA could be confirmed by SDS-PAGE analysis of outer membrane proteins extracted from cells grown in CAA medium (Fig. 2B). Growth in liquid cultures was monitored with a Bioscreen apparatus that allows the recording of the optical densities in microwell cultures. The addition of pyocin S2 alone or together with pyoverdine totally inhibited the growth of the sensitive strain W15Dec1, while the type I *fpvA* mutant could grow in the presence of the bacteriocin (results not shown). Again, complementation with either type I *fpvA*(*V46*) or type I *fpvA*(*I46*) restored the sensitivity to S2 (results not shown).

Expression of both forms of the type I *fpvA* gene confers the capacity for type I pyoverdine utilization and sensitivity to pyocin S2 on *P. aeruginosa* 7NSK2. *P. aeruginosa* 7NSK2 is a type II pyoverdine-producing strain (10, 18). Accordingly, *P. aeruginosa* 7NSK2 has the type II *fpvA* receptor gene and is sensitive to pyocin S3 (1, 10). An 7NSK2 type II *fpvA-fpvB*
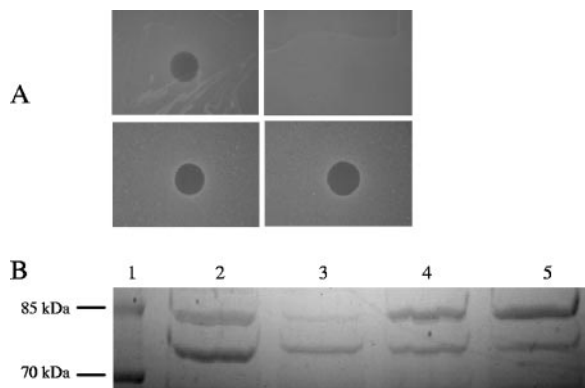
FIG. 2. Effect of the inactivation of type I *fpvA* in the pyocin S2-sensitive strain W15Dec1 and complementation in *trans* with the two type I *fpvA* genes (encoding V46 and I46). (A) Killing activity of pyocin S2 on wild-type W15Dec1 (top left panel), W15Dec1*fpvA*⁻ (top right panel), W15Dec1*fpvA*⁻ complemented with type I *fpvA(V46)* (bottom left panel), and W15Dec1*fpvA*⁻ complemented with type I *fpvA(I46)* (bottom right panel). (B) SDS-PAGE analysis of outer membrane proteins from CAA-grown wild-type W15Dec1 (lane 2), the W15Dec1 type I *fpvA* mutant (lane 3), the W15Dec1 type I *fpvA* mutant complemented with type I *fpvA(V46)* (lane 4), and the W15Dec1 type I *fpvA* mutant complemented with type I *fpvA(I46)* (lane 5). The band below the 85-kDa marker is FpvA. Lane 1, molecular mass markers.
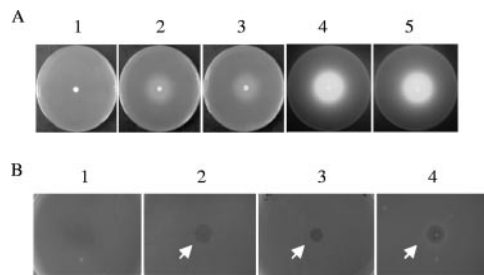

FIG. 3. Complementation of the type II pyoverdine 7NSK2*fpvA*⁻*fpvB*⁻ mutant strain. (A) Growth stimulation of 7NSK2*fpvA*⁻*fpvB*⁻ on CAA plates containing EDDHA around a type I pyoverdine-impregnated disk (panel 1) and the same mutant complemented with type I *fpvA(V46)* (panel 2) and type I *fpvA(I46)* (panel 3). Panels 4 and 5 are the plates from panels 2 and 3 shown under UV illumination to demonstrate the fluorescence due to type II pyoverdine production. (B) Results from an S2 pyocin killing assay of 7NSK2*fpvA*⁻*fpvB*⁻ (resistant; panel 1) and the same mutant complemented with type I *fpvA(V46)* (sensitive; panel 2) and type I *fpvA(I46)* (sensitive; panel 3). Panel 4 shows the killing of the sensitive indicator strain O:9 as a positive control.

double mutant is unable to utilize either type I ferripyoverdine (via FpvB) or type II ferripyoverdine because of the loss of the cognate receptor type II FpvA (13). Both type I *fpvA* genes (encoding V46 and I46) were cloned into the wide-host-range vector pBBR1*mcs*, and the resulting recombinant plasmids were introduced by conjugation into 7NSK2*fpvA*⁻*fpvB*⁻. In both cases, heterologous complementation with the type I *fpvA* gene conferred the capacity to grow in the presence of the high-affinity iron chelator EDDHA and type I pyoverdine (Fig. 3A). Surprisingly, the introduction of the heterologous type I *fpvA* gene not only conferred the ability to utilize externally added type I pyoverdine but also triggered the production of type II pyoverdine by 7NSK2, as judged by the intense fluorescent halo around the type I pyoverdine disk, suggesting that the type I FpvA receptor-FpvR antisigma factor interaction may signal type II pyoverdine production (Fig. 3A). Again, the expression of both variants of type I *fpvA* in *trans* rendered 7NSK2 sensitive to S2 (Fig. 3B).

DISCUSSION

The production of pyocins by different *P. aeruginosa* strains suggests that these bacteria use these killer proteins to eliminate related competitors which colonize the same niche (24, 26). Normally, strains are immune to killing by their own pyocin thanks to the production of an immunity protein that binds to the nuclease C-terminal domain of the killing protein and inhibits its activity (24). The gene for the immunity protein is cotranscribed with the gene encoding the killer protein, ensuring that there is balanced production of the two proteins. However, in two recent transcriptome analyses, one of the response to H_2O_2 and the other of the response to the SOS-inducing antibiotic ciprofloxacin, it was observed that exposure to both agents induced the transcription of the killer pyocin S2

but repressed the transcription of the immunity gene (3, 6). It has been proposed previously that pyocins which recognize ferripyoverdine receptors are driving the evolution of these siderophore receptors and, at the same time, the diversification of pyoverdines (34). The term "Darwinian positive selection" has even been proposed (35). Our results demonstrate unambiguously that type I FpvA is needed for pyocin S2 killing. The fact that some strains (including PA14) having the FpvAI receptor but not the S1-S2 immunity gene still resist killing by S2 is puzzling. One possibility is that these strains produce an immunity protein different from that for S1 and S2 but able to recognize the killing domain of S2. A BLASTtn analysis of the PA14 genome using the S2 immunity protein sequence as a query did not reveal the existence of any immunity protein with similarity to that for S1 and S2. Another possibility is that type I FpvA is needed as the primary receptor but that a second receptor is necessary to allow the entry of the pyocin into the cell. This is the case for some *E. coli* colicins, which recognize the vitamin B_{12} receptor BtuB as the primary receptor but enter the cell, depending on the colicin, via the TolC or OmpF outer membrane porin (5). Also, more insight is needed about the way the pyocins are translocated into the cell before we can fully understand why some strains are resistant to killing. During this investigation, other interesting observations concerning pyoverdine signaling were made. First of all, we could demonstrate that the expression of type I *fpvA* in a type II *fpvA* host, in which the alternative FpvB receptor for type I ferripyoverdine is not produced, is possible and that the receptor is functional since it confers the ability to grow in the presence of EDDHA and type I pyoverdine. Even more surprising is that type I FpvA can signal the production of pyoverdine in the type II strain 7NSK2, which means that, in this strain at least, type I FpvA can interact with FpvR as efficiently as the cognate type II FpvA. In conclusion, the results presented here confirm the prediction made by Smith et al. in 1992 that the pyoverdine receptor is the receptor for Sa, and we have demonstrated that pyocin Sa is identical to the previously described pyocin S2 (25, 31, 33). The future challenge will be to under-

stand how some strains apparently lacking an immunity gene and producing the type I S2 pyocin FpvA receptor can be resistant to killing by the S2 bacteriocin.

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