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The *Pseudomonas aeruginosa pirA* gene encodes a second receptor for ferrienterobactin and synthetic catecholate analogues

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Abstract

Actively secreted iron chelating agents termed siderophores play an important role in the virulence and rhizosphere competence of fluorescent pseudomonads, including *Pseudomonas aeruginosa* which secretes a high affinity siderophore, pyoverdine, and the low affinity siderophore, pyochelin. Uptake of the iron–siderophore complexes is an active process that requires specific outer membrane located receptors, which are dependent of the inner membrane-associated protein TonB and two other inner membrane proteins, ExbB and ExbC. *P. aeruginosa* is also capable of using a remarkable variety of heterologous siderophores as sources of iron, apparently by expressing their cognate receptors. Illustrative of this feature are the 32 (of which 28 putative) siderophore receptor genes observed in the *P. aeruginosa* PAO1 genome. However, except for a few (pyoverdine, pyochelin, enterobactin), the vast majority of *P. aeruginosa* siderophore receptor genes still remain to be characterized. Ten synthetic iron chelators of catecholate type stimulated growth of a pyoverdine/pyochelin deficient *P. aeruginosa* PAO1 mutant under condition of severe iron limitation. Null mutants of the 32 putative TonB-dependent siderophore receptor encoding genes engineered in the same genetic background were screened for obvious deficiencies in uptake of the synthetic siderophores, but none showed decreased growth stimulation in the presence of the different siderophores. However, a double knock-out mutant of ferrienterobactin receptor encoding gene *pfeA* (PA 2688) and *pirA* (PA0931) failed to be stimulated by 4 of the tested synthetic catecholate siderophores whose chemical structures resemble enterobactin. Ferric-enterobactin also failed to stimulate growth of the double *pfeA–pirA* mutant although, like its synthetic analogues, it stimulated growth of the corresponding single mutants. Hence, we confirmed that *pirA* represents a second *P. aeruginosa* ferric-enterobactin receptor. The example of these two enterobactin receptors probably illustrates a more general phenomenon of siderophore receptor redundancy in *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; Enterobactin; Catecholate siderophores; *pfeA*; *pirA*; TonB-dependent receptors

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium endowed with an extremely versatile metabolism, reflected in its ability to colonize a wide variety of

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habitats, from mammalian host to the rhizosphere of plants [1]. As for most organisms, iron is indispensable for survival of *P. aeruginosa*. Unfortunately, iron, despite being one of the most abundant elements in the earth crust, is rarely freely accessible and its acquisition demands significant adaptations from microorganisms. A common microbial strategy for iron acquisition is the production of low-molecular mass iron-chelating compounds named siderophores [2]. For Gram-negative bacteria, uptake of the ferrisiderophore complexes into the cell necessitates specialized receptors acting as gated porin channels that recognize and actively internalize the ferrisiderophore complexes [2]. As a rule, ferrisiderophore receptors recognize exclusively one iron–siderophore complex, but exceptions to this rule have been reported [3–5]. Since the outer membrane is devoid of energy sources, all these receptors rely on a conserved protein, called TonB, and two other proteins, ExbB and ExbC, also located in the inner membrane, to transduce energy generated in the cytoplasmic membrane to the receptor protein [2]. Although three TonB homologues have been described in *P. aeruginosa*, only TonB1 seems to be involved in the uptake of ferrisiderophores [6]. Just like outer-membrane porins, these receptor proteins are shaped of a large C-terminal domain of 22 antiparallel β-strands, which form a membrane spanning β-barrel [7]. What distinguishes TonB dependent receptors from porins is an additional domain known as 'cork' or 'plug' that blocks the β-barrel domain and by using energy transduced by TonB, allows selective uptake of siderophore/iron complexes [8].

P. aeruginosa secretes a high affinity siderophore pyoverdine (PVD), and another siderophore, pyochelin (PCH), which displays lower iron affinity compared to PVD [9,10]. Besides producing endogenous siderophores, *P. aeruginosa* has the capacity to take up and utilize numerous siderophores secreted by other microorganisms including those of other bacteria (aerobactin, enterobactin and its precursor 2,3-dihydrobenzoic acid and breakdown product *N*-(2,3-dihydrobenzoyl)-L-serine), pyoverdines/pseudobactin from other pseudomonads, cepabactin, fungal siderophores (deferrioxamines, dererrichrysin, deferrirubin, coprogen), synthetic chelators, (e.g. nitrilotriacetic acid) and naturally occuring chelators such as citrate and myo-inositol hexakisphosphate [10]. Not surprisingly, the *P. aeruginosa* PAO1 genome [11] counts no less than 32 genes with ferrisiderophore receptor gene signature [9]. Only three of them were previously matched with a siderophore ligand: *fpvA* (ferri-PVD uptake) [12], *fptA* (ferric PCH uptake) [13] and *pfeA* (ferrienterobactin uptake) [14]. Recently, we described a gene, *fpvB*, which encodes a second receptor for PVD [15]. The active transport of siderophore–iron complexes across the outer membrane of Gram-negative bacteria has caught the interest of scientists exploring possible novel concepts of anti-microbial drug delivery. One possible approach to overcome the problem of resistance in *P. aeruginosa* [16] lies in the synthesis of antibiotics conjugated with compounds active as siderophores [17]. Two different carrier concepts are currently under evaluation: making use of either a derivative of a natural siderophore or artificial synthetic siderophore entities [17]. Tests on several Gram-negative species have demonstrated the applicability of both type of conjugates, exhibiting minimal inhibitory concentrations (MICs) that are significantly lower (up to 100 times) compared to the associated free drugs. However, drug conjugates with synthetic siderophore analogues are often easier to produce [17] and may be designed for application against a broader range of species. Hence, while exploring the properties of synthetic siderophore analogues for active drug delivery it is important to map the receptors that mediate their uptake. An ideal siderophore drug carrier is taken up by several receptors in order to minimize the chances of resistance development.

Many pathogenic bacteria, including *P. aeruginosa*, have outer membrane receptors for heterologous transport of ferrienterobactin (FeEnt), a siderophore produced by enteric bacteria [14,18]. It has been suggested that *P. aeruginosa* can take up enterobactin via two distinct uptake systems, one of "high affinity" induced by enterobactin, the second of "low affinity" not induced by enterobactin [19]. The *pfeA* gene, encoding the high affinity enterobactin receptor, has been cloned and sequenced [14]. Synthetic analogues that mimic enterobactin, but change certain aspects of its chemistry were previously used to determine the structural feature of the siderophore that are important to its transport. These studies have shown that the iron binding centre contains the primary determinants of the uptake reaction and that replacement of the natural macrocyclic ring had little effect on ferrienterobactin transport [20].

We evaluated the siderophore properties of a number of synthetic catecholate siderophore analogues on *P. aeruginosa* and tried to map their receptors and confirmed earlier suggestions for the presence of two ferrienterobactin uptake systems in *P. aeruginosa* PAO1 and identified the second, low affinity ferric-enterobactin uptake mediating receptor as the product of *pirA*. Since both ferrienterobactin receptors are also involved in the uptake of several synthetic enterobactin analogues, they represent good candidate drug carriers.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

The different *P. aeruginosa* mutations in putative ferrisiderophore receptor genes used in this study are listed

Table 1
List of 36 *P. aeruginosa* PAO1 TonB-dependent receptors genes

Gene no.	Gene name	Identified ligand	Gene no.	Gene name
PA 2398	*fpvA*	Ferripyoverdine	PA 4675	
PA 4168	*fpvB*	Ferripyoverdine	PA 1302	
PA 4221	*fptA*	Ferripyochelin	PA 4837	
PA 2688	*pfeA*	Ferrienterobactin	PA 2911	
PA 0931*	*pirA*	Ferrienterobactin	PA 1922	*cirA*
PA 4710	*phuR*	Heme	PA 2289	
PA 3408	*hasR*	Heme	PA 0192	
PA 1910	*ufrA*		PA 0434	
PA 0674*	*pigC*		PA 0781	
PA 0470*	*fiuA*		PA 1365	
PA 4514*	*piuA*		PA 1613	
PA 1322*	*pfuA*		PA 2057	
PA 3901	*fecA*		PA 2089	
PA 2466	*optS*		PA 3268	
PA 0151			PA 2335	*optO*
PA 4897	*optI*		PA 4156	
PA 4675	*iutA*		PA 2590	
PA 1302	*hxuC*		PA 2070	

List of 36 *P. aeruginosa* PAO1 TonB-dependent receptors genes for which the corresponding knock-out mutants were engineered in an unmarked allelic *pvdD pchEF* mutant.

* Fur-regulated genes picked up by the SELEX technique [21].

in Table 1. *P. aeruginosa* wild-type and mutants were grown under conditions of good aeration at 37 °C either in Casamino acid medium (CAA, low iron medium) or LB medium. The ferrisiderophore growth stimulation assays were performed in CAA supplemented with 10 μM of the iron chelator ethylenediamine dihydroxyphenylacetic acid (EDDHA) and 200 μM dipyridil (in agar medium) or CAA with 5 μM EDDHA and 100 μM dipyridil (in liquid medium) for iron-limiting conditions. For ferrienterobactin stimulation assays, a wild-type *E. coli* strain (MC4100) producing enterobactin was grown in CAA medium plus 0.2% glucose during 48 h. The supernatant was collected after centrifugation and filter-sterilized. Another *E. coli* strain, H6876, an *entC* derivative of MC4100 was grown under the same conditions. This strain is unable to produce enterobactin. The supernatants of both *E. coli* strains (15% V/V) were added to LB-agar plates containing EDDHA and dipyridil as described in 2.1.

2.2. Siderophores utilization assay

The catecholate synthetic siderophore analogues are shown in Fig. 1. Petri dishes of CAA solid agar medium containing 10 μM EDDHA + 200 μM dipyridil were used. Two hundred microlitres of a 10^5 CFU/ml cell suspension of the mutant was spread on the medium surface. A sterile paper disc impregnated with 5 μl of 2 mM siderophore solution was placed on top of the agar plate. Siderophore usage was detected, after 1 day of incubation at 37 °C, as a halo of growth around the filter disc. For the utilization of enterobactin, LB-EDDHA-dipyridil agar plates were used which contained 15% (V/V) filter-sterilized supernatants of *E. coli* MC4100 (enterobactin producer) and H6876 (*entC* mutant of MC4100, unable to produce enterobactin). On these plates, 0.1 ml of dilutions of *P. aeruginosa* cells (from 10^8 to 10^3 CFU per ml) was inoculated and the plates incubated overnight at 37 °C.

2.3. Liquid growth stimulation assays

For more accurate analysis, growth was assessed in microtiter plates using a Bio-Screen C incubator (Life Technologies®). Briefly, the following protocol was used: pre-cultures (2–3 ml) were grown overnight in CAA medium. The next day the pre-cultures were used to inoculate in a 1:100 ratio 3 ml cultures in CAA medium, which were grown till $OD_{600} = 0.5$. Serial dilutions in CAA were performed to reach a final 1:5000 dilution. The following parameters were programmed to be executed by the apparatus: each well contains: 295 μl of (CAA + 5 μM EDDHA + 100 μM 2-2′dipyridil) with 5 μl of 2 mM of the to be tested siderophores and 5 μl of DMSO for control wells (solvent used to dissolve the siderophores); shaking for 30 s every 3 min, absorbance measured every 20 min at 600 nm and temperature at 37 °C.

3. Results

3.1. Mutants in putative ferrisiderophore receptor genes in P. aeruginosa *PAO1*

A siderophore-free background was created in *P. aeruginosa* PAO1 by making unmarked deletions in *pvdD* (pyoverdine biosynthesis) and *pchEF* (pyochelin biosynthesis) [15]. Candidate siderophore receptor genes of *P. aeruginosa* PAO1 were originally picked up by a cycle selection procedure to identify iron repressed genes that are directly regulated by the Ferric Uptake Regulator (Fur) [21,22]. Five of these genes were found to be similar to known siderophore receptor genes (Table 1). More candidate siderophore receptor genes were counted in the completed *P. aeruginosa* PAO1 genome sequence (http://www.pseudomonas.com) [9,11]. No less than 36 ORFs carry the signature of TonB dependent receptor encoding genes. Four of them are the previously identified ferrisiderophore receptor genes for respectively ferri-pyoverdine (*fpvA*, PA2398; *fpvB*, PA4168), ferri-pyochelin (*fptA*, PA4221) and ferrienterobactin (*pfeA*, PA2688). Also included are the TonB dependent receptors involved in haem-uptake encoded by *phuR* (PA4710) and *hasR* (PA3408) [23]. In the earlier created siderophore-free mutant (*pvdD pchEF*) of *P. aeruginosa* PAO1, 'candidate' siderophore receptor genes, 36 in total, were

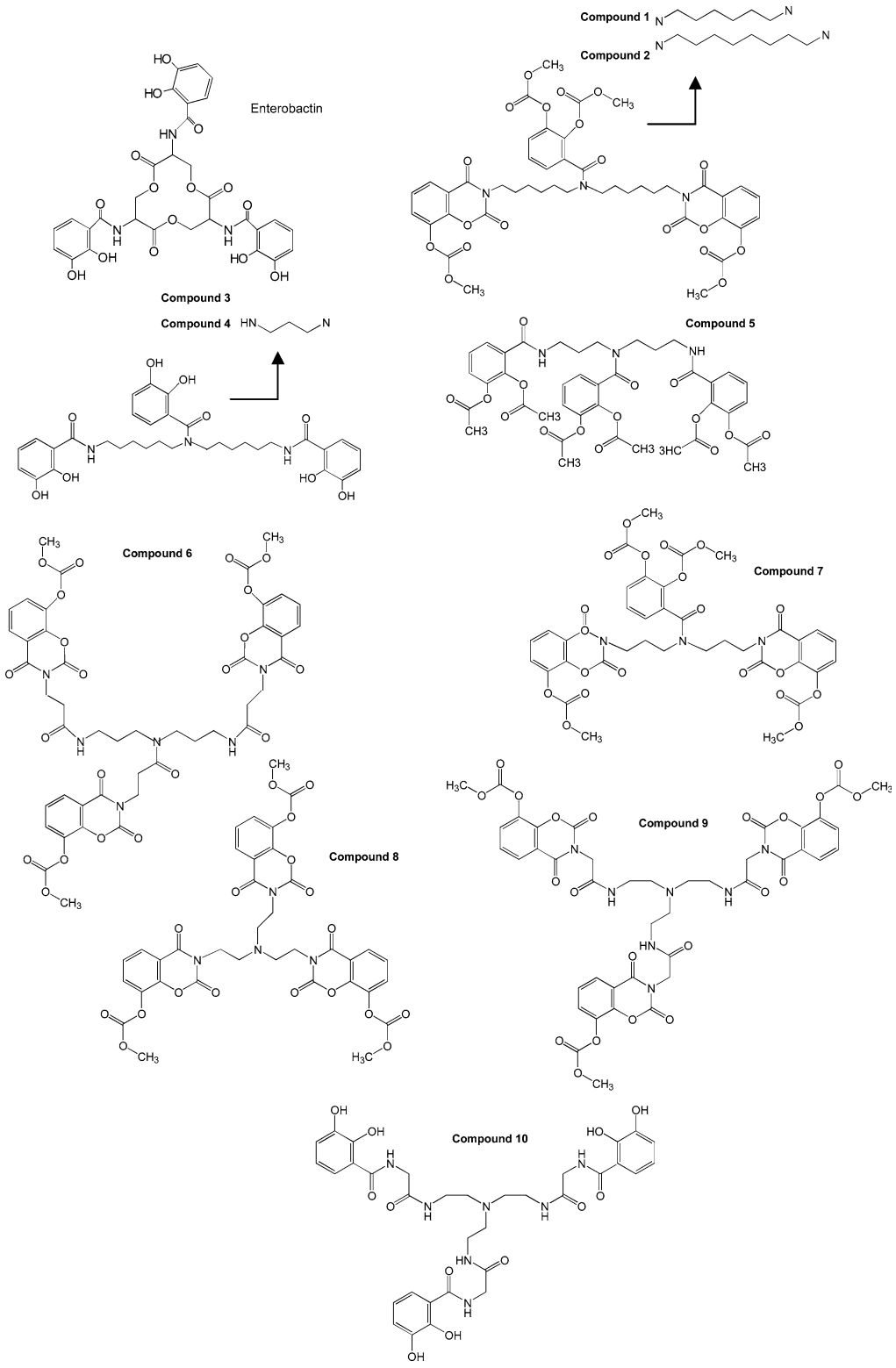

Fig. 1. Structures of enterobactin and the Tris-catecholate synthetic siderophores used in this study.

knocked-out by allelic exchange with interrupted copies of their gemonic alleles [15]. All mutants were analyzed by PCR in order to confirm the presence of the unmarked deletion [15].

Since *P. aeruginosa* can take up enterobactin via two distinct uptake systems [19], an additional mutant was engineered with simultaneously knocked-out *pfeA* (PA2688), the ferrienterobactin receptor, and *pirA*

(PA0931), the candidate ferrisiderophore receptor gene within the PAO1 genome with the highest similarity to *pfeA* (72% similarity between their translation products).

3.2. Utilization of synthetic catecholates by the different TonB-dependent mutants

The *P. aeruginosa* PAO1 *pvdD pchEF* mutant carries deletions in genes for PVD and PCH synthesis and is therefore unable to grow in an iron restricted situation created by the presence of both a ferric iron chelator (10 μM EDDHA) and ferrous iron chelator (200 μM 2-2′-dipyridil). We aimed to select synthetic siderophore analogues that are capable of stimulating growth of this siderophore deficient mutant in presence of EDDHA and dipyridil, which, in other words function as xeno-siderophores that can be assimilated by *P. aeruginosa*. Ten "catecholate" compounds were used [24] (see Table 2). The "catecholates" represent either the free forms (compounds 3, 4 and 10) and the protected forms (compounds 1, 2 and 5–9). There were 2 types of protected forms, form "a" as aliphatic acyloxy group (compound 5), form "b" as heterocyclic benzoxazine residue (compounds 6 and 8) and mixed forms of both (compounds 1, 2 and 7). The basic structures for the catecholates were either linear (compounds 1–7) or tripodal (compounds 8–10). We assume, that the protected forms can split off to the free catecholates under physiological conditions since obviously only these structures can form iron complexes. Additionally it should be mentioned that the antibiotic conjugates of the protected catecholates are active as antibacterials via uptake by ferrisiderophore transport pathways [24–27].

Table 2
Summary of the results of growth stimulation tests

Catecholate compound	Siderophore	*pvdD pchEF*	*pvdD pchEF*		
			pfeA	*pirA*	*pfeA pirA*
1	HKI 9824013	++	++	++	++
2	HKI 9824014	++	++	++	++
3	HKI 9824030	++	++	++	++
4	HKI 9824043	++	++	++	++
5	HKI 9824080	++	++	++	++
6	HKI 9924127	++	++	++	++
7	HKI 9824032	++	++	++	–
8	HKI 10024023	++	++	++	–
9	HKI 10024024	++	++	+	–
10	HKI 10024025	++	++	+	–

Summary of the results of growth stimulation tests peformed with the synthetic siderophore analogues on the *pvdD pchEF* siderophore production deficient background strain, and the mutants in *pfeA*, *pirA* and the double *pfeA pirA* knock-out, all created in the *pvdD pchEF* background. Strong growth stimulation (++), weak/delayed growth stimulation (+), no growth stimulation (−). The catecholate synthetic siderophore structures are presented in Fig. 1.

All 10 catecholate compounds stimulated the growth of the siderophore-deficient *P. aeruginosa* PAO1 *pvdD pchEF* mutant under these conditions of extreme iron limitation. Although each of the putative TonB-dependent receptor genes of *P. aeruginosa* PAO1 had been inactivated, none of the 36 single mutants failed to be stimulated by any of the selected synthetic siderophores, suggestive of a redundancy in siderophore uptake systems in *P. aeruginosa*. Interestingly, the synthetic enterobactin analogues 7–10 stimulated single knock-out mutants of *pfeA* (PA2688), the high affinity enterobactin receptor gene, and PA0931 (*pirA*) its closest homologue within *P. aeruginosa* PAO1, but failed to stimulate a mutant with both genes inactivated (Table 2).

3.3. Growth stimulation by enterobactin

Since we did not have purified enterobactin, we looked at the growth stimulation conferred by the addition of cell-free supernatant from a culture of wild-type *E. coli* MC4100 (enterobactin producer) and an *entC* derivative from the same strain (unable to produce enterobactin) grown under iron-limiting conditions. As shown in Fig. 2, the supernatant from MC4100 stimulated the growth of the *P. aeruginosa pvdD pchEF* mutant (Fig. 2(a)), *pvdD pchEF pfeA* (Fig. 2(b)) and *pvdD pchEF pirA* (Fig. 2(c)), but not of the mutant *pvdD pchEF pfeA pirA* (Fig. 2(d)). As could be expected, the supernatant from the *entC* mutant could not stimulate the growth of any of these *P. aeruginosa* strains (results not shown). This observation confirms that PA0931 (*pirA*) serves as second ferrienterobactin receptor next to *pfeA*.

3.4. Growth kinetics of pfeA and pirA in response to stimulation by catecholates

The previous growth stimulation tests were performed with siderophore impregnated filter-discs on CAA-agar medium containing EDDHA and dipyridil. In order to determine a hierarchical order between the PfeA and PirA receptors in affinity for the different enterobactin-like ligands, we kinetically measured growth responses of the *pfeA* and *pirA* mutants towards the different synthetic enterobactin analogues in EDDHA- and dipyridil-containing liquid CAA cultures. When compared to the *pvdD pchEF* strain and the single *pfeA* mutant in the same genetic background, a delayed growth response of the *pirA* mutant was observed towards compounds 9 (data not shown) and 10 (Fig. 3) but not to 7 (Fig. 3) or 8 (result not shown). In contrast to ferrienterobactin which is taken up preferentially by PfeA [14,19] the iron complexes with the synthetic siderophore analogues 9 and 10 are more efficiently taken up by PirA.

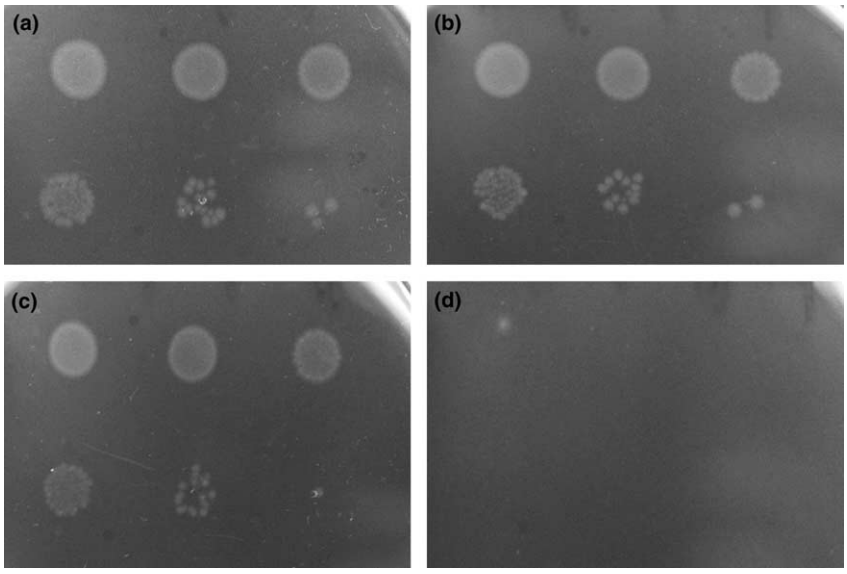

Fig. 2. Growth of *P. aeruginosa pvdD pchEF* (a), *pvdD pchEF pfeA* (b), *pvdD pchEF pirA* (c) and *pvdD pchEF pfeA pirA* (d) in LB-agar plates containing EDDHA, dipyridil and 15% (V/V) of filter-sterilized culture supernatant of wild-type *E. coli* MC4100 (producer of enterobactin). From left to right, starting from the top, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 *P. aeruginosa* cells. No growth was observed when the plates contained 15% (V/V) supernatant from an *E. coli entC* mutant which does not produce enterobactin (results not shown).

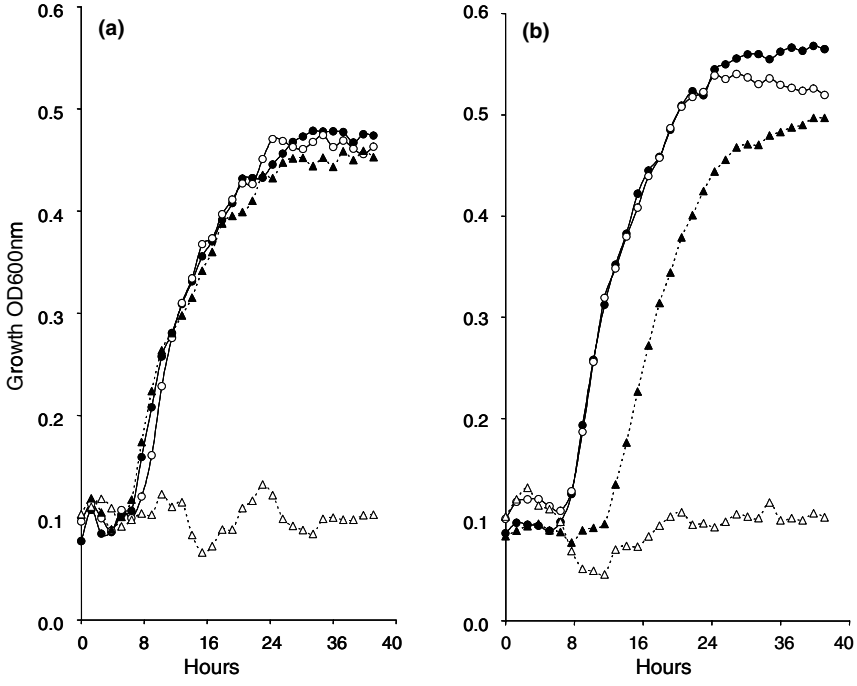

Fig. 3. Growth stimulation of the *pvdD pchEF* (●), *pvdD pchEF pfeA* (○), *pvdD pchEF pirA* (▲), and the *pvdD pchEF pfeA pirA* (△) mutants by compound 7 (left) and compound 10 (right) in the presence of EDDHA and dipyridil. The values on the *Y* axis correspond to the OD at 600 nm. Only one representative growth curve out of three separate experiments is shown.

4. Discussion

P. aeruginosa has the ability to use siderophores secreted by other species in order to fulfil its needs for iron [9,10]. This capacity of xenosiderophore usage illustrates the importance of iron acquisition in microbial ecology. Although, more than thousand different siderophore compounds have been identified to date, they are usually constructed with the same basic elements consisting of catecholates, hydroxamates or carboxylates, preferentially in a tri-bidentate iron complexing conformation. This inspired researchers to create novel, synthetic

siderophore compounds based on these naturally conserved themes. Shared chemo-structural motifs between synthetic and natural siderophores, allow their ferrisiderophore complexes to be taken up by the same cognate receptors. Catecholate derivatives were generated with high siderophore activities in strains of *P. aeruginosa* and *E. coli* [25]. β-Lactam conjugates of these siderophores showed enhanced antibacterial activities which could be attributed to the active iron uptake routes used by the conjugates to penetrate the bacterial cells [24–27]. The 10 synthetic catecholate siderophores used in this study stimulated the growth of a siderophore-negative *P. aeruginosa* under conditions of strong iron limitation, indicating that these siderophores had sufficient iron binding affinity to displace iron from EDDHA and dipyridil and could be assimilated by the cell. The fact that the growth of all siderophore-negative mutants with a single receptor gene inactivation could be stimulated by the 10 compounds suggests the presence of at least two receptors for a given ferrisiderophore. Such receptor redundancy has interesting implications for the use of synthetic xenosiderophore analogues as drug carriers. Indeed, when a siderophore-drug conjugate can penetrate the cell via several independent receptors, the risk of resistance development is significantly reduced. Receptor redundancy, on the other hand, complicates the mapping of receptor genes by a knock-out approach since the dysfunctional receptor phenotype can be masked by another receptor recognizing the same ligand. We recently demonstrated the applicability of the idea that receptor pairs with high sequence similarity mediate the uptake of the same ligand [15], providing a rational base for engineering multiple receptors knock-out mutants. The *pfeA* gene, encoding the high affinity enterobactin receptor, has been cloned and sequenced [14]. Nonetheless, PfeA-deficient mutants display growth, albeit reduced, in an enterobactin supplemented, iron-restricted minimal medium [19,28]. The best candidate for a second ferrienterobactin receptor is the product of PA0931, dubbed *pirA*, which displays substantial similarity with *pfeA*. With the receptor mutants engineered in a pyoverdine and pyochelin-free background, we unambiguously confirmed that *P. aeruginosa* indeed counts two ferrienterobactin transporting receptors, PfeA and PirA. Therefore the situation in *P. aeruginosa* is similar to the situation in *Salmonella enterica* where two receptors, FepA and IroN mediate the transport of ferrienterobactin [29]. Interestingly, we could not detect any difference in growth stimulation by ferrienterobactin of the *pfeA* or *pirA* mutant (Fig. 2), which seems to be in contradiction with the results obtained before [19,28]. This could be due to the difference of genetic background since we used a mutant of *P. aeruginosa* which is unable to produce either pyoverdine of pyochelin. Growth kinetics of the mutants suggested that two of the synthetic enterobactin analogues tested are preferentially transported by the PirA receptor in contrast to enterobactin for which PfeA acts as the high affinity receptor. It is therefore likely that PirA transports another yet unknown natural siderophore different from enterobactin as its primary substrate. Another enterobactin-like siderophore, bacillibactin, is produced by the Gram-positive *Bacillus subtilis* [30] and it would be interesting to look at the transport of this ferrisiderophore using the same set of mutants described in this study. Another interesting question for the future is to understand why only compounds 7–10 are taken by *fepA* and *pirA* like enterobactin. It has to be mentioned that compounds 8–10 are tripodal while compound 7 is the only linear catecholate analogue which is taken up by these two receptors. Also, it would be interesting to discover which TonB-dependent receptors mediate the transport of compounds 1–6 since their growth stimulation properties are not affected by the *fepA pirA* mutations.

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