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ORIGINAL PAPER

Chemical characterization and ligand behaviour of *Pseudomonas veronii* 2E siderophores

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Abstract

Siderophores are low-molecular weight ligands secreted by bacteria as a survival strategy in Fe(III)-lacking environments. They bind not only Fe(III), but Co(II), Zn(II), Mn(II), Ni(II), Ga(III) as a detoxification alternative. The synthesis, purification and characterization of siderophores produced by *Pseudomonas veronii* 2E were evaluated to be applied in future environmental technologies. Optimal production was obtained in Fe(III)-free M9-succinate at 25 °C, 40 h and pH 6.9. Siderophores were chemically characterized as hydroxamate and catechol mixed-type. Spectroscopic analysis indicated their belonging to the pyoverdine family, behaving as ligand to Cd(II), Zn(II), Cu(II), Ni(II) and Cr(III), which promoted siderophoregenesis during growth. Siderophore-Cd(II) complexation was studied by electrochemical monitored titration revealing one family of moderate-strength binding sites. Mass spectral analysis evidenced the secretion of a variety of molecules (molecular mass ca.1200 u). Non pathogenic *Pseudomonas veronii* 2E siderophores represent a safe alternative for the concrete application of environmental technologies and clinical procedures.

Keywords Siderophores · *Pseudomonas veronii* 2E · Pyoverdine · Complexing capacity · Metal ligand · Cd(II) removal

Introduction

Siderophores are low molecular weight molecules that chelate Fe(III), forming soluble complexes that can then be incorporated by organisms to provide iron (Saha et al. 2016). Iron is an essential micronutrient in almost all microorganisms and is abundant in the environment. At neutral pH values, Fe(II) is soluble and available to be incorporated by bacteria, fungi and plants mediated by divalent metal transporters (Miethke and Marahiel 2007). However, in the presence of oxygen or enzymes, Fe(II) is easily oxidized to Fe(III) forming insoluble compounds in water at neutral to basic pH. As consequence, the soluble and bioavailable ferrous ion concentration remains in the order of 10^{-10} to 10^{-18} mol L^{-1}, whereas the necessary concentration for the microbial development is 10^{-6} mol L^{-1} (Boukhalfa and Crumbliss 2002; Johnstone and Nolan 2015). Hence the biological production of siderophores is particularly active in environments with iron limitations. These compounds are divided into four main families depending on the characteristic functional group that interacts with Fe(III), i.e., hydroxamate, catechol, carboxylate or a mixed group. For example, fluorescent pseudomonads produce siderophores corresponding to a mixed-type hydroxamate and catechol group, usually called pyoverdines (Miethke and Marahiel 2007). These yellow–green siderophores bind Fe(III) with high affinity at neutral pH values (Log K′f ~ 24–30.8) (Albrecht-Gary et al. 1994; Cézard et al. 2015; Visca et al. 2007). Pyoverdines present three distinct structural parts: the conserved fluorescent *chromophore* (derived from dihydroxyquinoline) attached via its carbonyl group to a variable *peptide chain* composed of 6–14 amino acids and a *side chain* corresponding to a carboxylic acid derivative (Fuchs et al. 2001; Meyer et al. 2002).

Siderophores also play an important role in the expression of virulence (Saha et al. 2012), development of biofilms (Banin et al. 2005), quorum sensing (Guan et al. 2001) and

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plant growth-promoting properties of rhizobacteria (PGPR) (Crowley 2006; Masalha et al. 2000). They have also relevant applications in medical field, i.e., as in delivery of antibiotics by carrying drugs into antibiotic-resistant bacteria cells (Braun et al. 2009; Huang et al. 2013; Mollmann et al. 2009). These microbial metabolites have received more attention in recent years because of their potential applications in the environmental area, i.e., as biopesticides or biosensors for ferric ion, in phytoremediation and bioremediation of toxic metals (Ahmed and Holmstrom 2014; Barrero et al. 1993; Saha et al. 2012, 2016). Although the primary function is to chelate Fe(III), they also form complexes with other essential or non-essential metals as Cd(II), Cu(II), Mn(II), Zn(II), Co(II), Ga(III), Al(III), In(III), Th(IV), U(IV) and Pu(IV) (Braud et al. 2009b; Johnstone and Nolan 2015; Nair et al. 2007; Mehri et al. 2012; Schalk et al. 2011; Visca et al. 1992). This ability primarily depends on their ligand functionalities and several studies have reported that siderophores impact in the mobility of these metals in the environment (Ahmed and Holmstrom 2014; Saha et al. 2012). In this way, siderophores become a useful and environmentally friendly tool for metal removal.

The aim of this work is focused on non reported siderophores of *Pseudomonas veronii* 2E, in particular production, chemical characterization and the evaluation of the ligand behaviour with Cd(II), Cu(II), Ni(II), Cr(III) and Zn(II) as a potential tool for environmental technologies. Being *P. veronii* 2E a non-pathogenic strain, the production of the secreted siderophores and their application in environmental technologies are promising, ecofriendly and safe enough to be implemented both in metal removal and metal recovery processes.

Materials and methods

Microorganisms

Pseudomonas veronii 2E is an autochthonous microorganism isolated from sediments associated to the Reconquista River basin (Buenos Aires Metropolitan Area). *P. veronii* 2E was identified by 500 bp 16S r-RNA gene sequencing and is able to retain Cd(II), Zn(II) and Cu(II) and biotransform Cr(VI) from aqueous systems, as described in previous studies (Alessandrello and Vullo 2016; Garavaglia et al. 2010; Méndez et al. 2011; Vullo et al. 2008). The optimal growth conditions for *P. veronii* 2E are 32 °C and pH 6–7.

Detection of siderophores

Different assays were used for siderophore detection. To evaluate siderophore production in the culture media three tests were carried out: absorbance measurement at $\lambda = 404$ nm (Kumar and Dube 1991; Merja and Tiina 1994; Meyer and Abdallah 1978), the universal CAS-agar assay and CAS-liquid assay. For all the determinations, cells were separated by centrifugation (7000×*g*, 15 min) and the supernatants were filtered through cellulose nitrate membrane filters (0.45 µm diameter).

For Chrome Azurol S (CAS) assay, CAS-agar (100 mL) was prepared according to Schwyn and Neilands (1987) with modifications. 7.5 mL of 2 mmol L^{-1} Chrome Azurol S were mixed with 1.5 mL iron solution (1 mmol L^{-1} $FeCl_3 \cdot 6H_2O$, 10 mmol L^{-1} HCl). Under stirring, 6.0 mL of 10 mmol L^{-1} cetyltrimethylammonium bromide (CTAB) solution were added and the resulting blue solution was kept at 50 °C. In addition, buffer solution was prepared using 0.6 g of PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] dissolved in 85 mL in ultrapure water (18 MΩ cm, Millipore) adjusting a final pH to 6.8 using 6 mol L^{-1} KOH. To this solution, 1.5 g agar–agar was added and then heated at 50 °C to dissolve it. The blue and the agar solutions were finally mixed, and Petri dishes (10 cm diameter) were prepared with 30 mL of CAS-agar. After gelling, 5 mm diameter holes were made and dried 1 h at 32 °C. Each hole was filled with 75 µL culture supernatant. A non-inoculated medium as negative control and cell-free supernatant from *Pseudomonas aeruginosa* PA01 cultures (Cox and Graham 1979; Visca et al. 1992) as positive control was used. After 1-day incubation at room temperature, the diameter of each orange halo formed around the holes was measured. Siderophore production was expressed as relative diameter (Eq. 1).

$$\text{Relative diameter} = \left(\text{diameter}/\text{OD}_{600\text{nm}}\right)_{P.\ veronii\ 2E} \Big/ \left(\text{diameter}/\text{OD}_{600\text{nm}}\right)_{P.\ aeruginosa\ PA01} \quad (1)$$

CAS-liquid assay was also used, because of its higher sensitivity and faster response compared to the CAS-agar test. This assay was performed to evaluate the production of siderophores by *P. veronii* 2E in M9S (see composition below) at different temperatures and pH values. CAS-liquid (100 mL) was prepared according to Schwyn and Neilands (1987) with modifications. 7.5 mL of 2 mmol L^{-1} Chrome Azurol S were mixed with 1.5 mL iron solution (1 mmol L^{-1} $FeCl_3.6H_2O$, 10 mmol L^{-1} HCl). Under stirring, 6.0 mL of 10 mmol L^{-1} CTAB solution were added resulting in a blue solution. Buffer solution was prepared using 0.6 g of PIPES in 85 mL ultrapure water, and pH was adjusted at 6.8 using 6 mol L^{-1} KOH. This solution was added to the blue solution and the mix was completed to a final volume of with ultrapure water. 1.5 mL CAS-liquid solution was mixed with 1.5 mL of cell-free supernatant and absorbance was measured at 630 nm after 30 min. A non-inoculated medium was used as negative control. Results were expressed as % siderophore (Eq. 2).

$$\%\ \text{siderophore} = \left[(\text{Ar} - \text{As})/\left(\text{Ar} \times \text{OD}_{600\text{nm}}\right)\right] \times 100 \quad (2)$$

Ar represents the absorbance at 630 nm of reference (CAS-liquid solution with non-inoculated medium) and As the absorbance at 630 nm of the sample (Sayyed et al. 2005).

Production of siderophores

First, siderophore production was tested in different culture media. To evaluate the effect of carbon and nitrogen sources, an enriched medium PYG (g L^{-1}: 2.5 peptone, 1.25 yeast extract, 0.5 glucose) and a minimal basal Fe-free medium M9 (g L^{-1}: 7.3 K_2HPO_4, 3.0 KH_2PO_4, 6.6 NH_4Cl, 3.3 NaCl, pH adjusted at 7 before autoclaving) supplemented with 5.0 g L^{-1} succinate (M9S) or 14 g L^{-1} glycerol (M9G) were used. Cultures were grown in batch system, 40 h at 25 °C with constant shaking at 120 rpm in 125 mL Erlenmeyer containing 10 mL of medium and 1 mL inoculums, previously obtained in the same conditions for 24 h (exponential phase). All material was cleaned in 6 mol L^{-1} HCl ensuring iron removal. Ultrapure water was used to rinse the glass material and for the preparation of culture media and all solutions. A non-inoculated medium was used as negative control in each case.

To evaluate the temperature effect, *P. veronii* 2E was grown in M9S below optimal growth temperature (20 °C and 25 °C), optimal growth temperature (32 °C) and above optimal growth temperature (38 °C). First, *P. veronii* 2E was pre-cultured in 25 mL of the medium at 120 rpm until late exponential phase. A total of 1 mL culture was transferred into another 10 mL M9S and incubated in the same conditions as the pre-culture for 40 h. A non-inoculated medium was used as negative control.

To check the effect of pH on siderophore production, the *P. veronii* 2E was grown in M9S by adjusting the pH before inoculation. Four 0.01 mol L^{-1} buffer solutions below and above the optimal growth pH were used: pH 5.8, MES (2-[*N*-morpholino]ethanesulfonic acid); pH 6.2, PIPES [Piperazine-*N,N*′-bis(2-ethanesulfonic acid)]; pH 6.9, $HPO_4^{2-}/H_2PO_4^-$ and pH 7.8, HEPES (*N*-[2-hydroxyethyl] piperizine-*N*′-[2-ethanesulfonic acid]).

Finally, to study the production of siderophores in time, *P. veronii* 2E was grown in M9S under the optimal pH and temperature conditions. Biomass and siderophore production were monitored up to 40 h.

In all cases, biomass was estimated from OD at 600 nm.

Chemical characterization of molecules

Catechol and hydroxamate groups of the siderophores produced by *P. veronii* 2E were qualitatively detected. Csáky assay (Csáky 1948) and its modification introduced by Gillam et al. (1981) were used to detect hydroxamate motives. Catechol-type siderophores in supernatants were detected by Arnow (1973) and modified Rioux assays (Rioux et al. 1983). Arnow test is not sensitive enough when positions 3 and 4 are substituted by catechol groups. In such case the Rioux assay is recommended. 2, 3-dihydroxybenzoic (DHBA) and *P. aeruginosa* PA01 culture supernatant were used as a positive controls and cell-free medium was used as negative control.

Effect of metals on siderophore biosynthesis assays

To evaluate the effect of metal ions in siderophore biosynthesis, 10 µmol L^{-1}, 100 µmol L^{-1} and 500 µmol L^{-1} of Zn(II), Cd(II), Ni(II), Cu(II) and Cr(III) as final concentrations in M9S medium were used. For each metal, a concentration below the minimum inhibitory concentration (MIC) was chosen according to previous studies (Garavaglia et al. 2010; Vullo et al. 2008). The initial pH value was 6.0 to ensure metal bioavailability.

10 mmol L^{-1} metal stock solutions were prepared with: $CdCl_2{\cdot}H_2O$ (maximum content Fe(III) 0.001%), $ZnSO_4{\cdot}7H_2O$ (maximum content Fe(III) 0.001%), $Cu(NO_3)_2.3H_2O$ (maximum content Fe(III) 0.002%), $NiSO_4{\cdot}6H_2O$ (maximum content Fe(III) 0.001%) and $CrCl_3{\cdot}6H_2O$ (maximum content Fe(III) 0.001%) and sterilized by filtration through 0.45 µm diameter-cellulose nitrate membrane.

Bacterial growth and siderophore production were respectively registered by measuring OD at 600 nm and absorbance at 404 nm as explained above.

Screening of metal–siderophore interactions

Sterile culture supernatants containing siderophores were used to study metal-siderophore interactions. For that purpose, the supernatants were incubated 3 h with 1, 10 or 100 µmol L^{-1} of Zn(II), Cd(II), Ni(II), Cu(II) or Cr(III). The interaction was determined by spectrophotometric scanning ($\lambda = 300$–480 nm), using non-inoculated M9S as control.

Siderophores as Cd(II) ligand

Siderophore separation

To separate and purify siderophores for ligand behavior evaluation, they were produced in 1000 mL M9S, 40 h, 25 °C, pH 6.9. After growth, cells were separated by centrifugation (7000×*g*, 15 min) and the supernatants were filtered through cellulose nitrate membrane to eliminate cells (1.2 µm pre-filter and 0.45 µm filter diameters respectively). 2.2 volumes of cold ethanol were added to the filtered solution and kept overnight at 4 °C. The pellet, containing extracellular polymeric substances (Ferreira et al. 2017), was discarded and the supernatant was concentrated to dryness using vacuum evaporator. The resulting pellet was dissolved in 30 mL of ultrapure

water (pH 4). The solution was incubated for 6 h with of XAD-2 resin (Amberlite, XAD-2) previously stabilized in H_2O–CH_3OH 1:1. After incubation, resin loaded with siderophores was placed in a column and washed with 3 volumes of ultrapure water. Siderophores were eluted with H_2O–CH_3OH 1:1; partially purified siderophores present in the solvent were again dried in the vacuum concentrator. The solid was dissolved in 10 mL ultrapure water and incubated 1 h with 0.5 g ion exchange resin (Chelex®100) to remove any metal traces interacting with the binding sites. After incubation, the resin was removed and the final supernatant was used to study the interaction with Cd(II).

Determination of ligand concentration and conditional complexation constants

The ability of siderophores to complex Cd(II) was studied using an electrochemically monitored titration where the non-bound metal was measured after the addition of increasing amounts of a standard Cd(II) solution (10 ppm or 100 ppm).

400 µL of a purified siderophore solution was added to 15 mL of 0.01 mol L^{-1} buffer MES (pH 6.2) containing 0.05 mol L^{-1} KNO_3 as supporting electrolyte. The pH value was chosen to minimize siderophore decomposition. After each Cd(II) addition, the mixture was stirred for 5 min for equilibration (determined in preliminary experiments). Titrations were monitored by Square Wave Anodic Stripping Voltamperometry (SW-ASV), applying −0.75 V during 2 min to achieve Cd(II) preconcentration. Each measurement was performed twice with good reproducibility. A 663VA polarographic stand and an Autolab PGStat10 potentiostat (Metrohm–Ecochemie) were used, both instruments controlled by the GPES software. A three-electrode cell containing a mercury drop electrode was used to perform the titrations at (25 ± 1) °C under N_2 atmosphere. All potentials were referred to a Ag/AgCl reference electrode (3 mol L^{-1} KCl).

The calculation of the number of ligand families and conditional complexation constants, K′, were carried out using Scatchard and Ružić linearizations (Ružić 1996; Scatchard 1949).

55 mg of fine particulate diatomite samples were incubated in presence of different Cd(II) concentrations (1.2–88 µmol L^{-1}) at pH 6.2, 120 rpm, 24 h and 25 °C. After incubation, diatomite samples were separated by centrifugation (7000×*g*, 15 min) and Cd(II) concentration in the supernatant was determined by standard addition using SW-ASV. The calculation of conditional complexation constants, K′, was carried out using Scatchard linearization (Scatchard 1949) and their values were compared to the corresponding K′ obtained for siderophore-Cd(II) complex.

Siderophore structural analysis

Siderophore purification

The methods of Meyer et al. (1997) were used with minor modifications. A partial purification was carried out in 500 mL of culture supernatants (M9S, 40 h, 25 °C, pH 6.9) as explained above. The resulting pellet was dissolved in 50 mL ultrapure water and 0.5 g $FeCl_3 \cdot 6H_2O$ (adjusted to pH 4–5 with 6 mol L^{-1} HCl) was added and incubated for 24 h. After this step, the solution was centrifuged (7000×*g*, 15 min) and the supernatant was retained with a characteristic brown color observed as a consequence of the siderophore-Fe(III) interaction.

Then, siderophore-Fe(III) solution was extracted with three volumes of phenol:$CHCl_3$ (1:1). The organic fraction was further treated with one volume of diethyl ether and 0.2 volume of ultrapure water. The water-diethyl ether extraction was repeated until no brown color was observed in the organic fraction. The aqueous phases were pooled together and washed three times with diethyl ether to remove residual phenol and concentrated as previously described. The obtained solid was dissolved in ultrapure water to a 1.7% (w/v) final concentration.

For a deep purification, a Sephadex G-25 column was prepared with 3 mL resin and equilibrated with H_2O–CH_3OH (1:1). Then, a 250 µL sample obtained above was loaded onto the column and washed with 3 volumes of ultrapure water (pH 4). The sample was then eluted with H_2O–CH_3OH (1:1). Fractions containing the complex were pooled together and dried by using rotary vacuum evaporator. The residual pellet was dissolved in 500 µL of water and 250 µL were loaded on a column with 3 mL resin RP-18 [previously stabilized with H_2O–CH_3OH (1:1)]. The column was washed with 3 volumes of water (pH 4–5) and the complex was eluted with water–methanol (1:1). Colored fractions were pooled and dried.

Mass spectra

Mass spectrometry was used to determine the molecular weight of purified siderophores. This analysis was performed by a nanoHPLCMS with Orbitrap technology. Separation of the siderophore-Fe(III) complexes present in the sample was carried out in an EASY-Spray C18 column (2 µm, 100 Å, 50 µm × 150 mm). For this analysis, detector voltage was fixed at 3.5 kV and sample ionization was performed by electrospray; fragments were analyzed based on their mass-to-charge ratio (m/z). Mass spectra were recorded in the m/z range 500–1500. Molecular ion of mass 1242.4 u was fragmented again to obtain structure characterization of siderophores.

Results

Production of siderophores by *P. veronii* 2E

Tests for siderophore production of cell-free supernatants in CAS-agar plate gave a positive result for *P. veronii* 2E grown in M9S and M9G while a negative result was observed when using PYG (Fig. 1a). The best medium formulation for the production of siderophores was M9S (0.63 ± 0.07 relative diameter). The non-inoculated media were negative for the assay.

To evaluate the effect of temperature on siderophore production, bacterial growth was evaluated at 20 °C, 25 °C, 32 °C and 38 °C in M9S medium. The siderophore secretion yield was calculated by Ec.2 (see Materials and Methods). Results in Fig. 1b indicate that the production of siderophores was clearly affected by temperature. Optimal production was obtained at lower temperatures. For next siderophore assays, 25 °C was selected because of the convenience of keeping it constant.

To study the effect of pH, siderophore secretion was evaluated in the range 5.8–7.8. As already mentioned, bacteria need Fe(III) concentrations around 1 µmol L^{-1} and secrete siderophores as a strategy for their uptake and incorporation under soluble iron scarcity conditions. The soluble Fe(III) concentrations at different pH values are shown in the Online Resource 1. Soluble Fe(III) concentration was calculated considering the initial Fe(III) concentration in M9S medium, solubility and the formation constants of the most important complexes currently present in aqueous media (Martell and Smith 2010). Figure 1c shows an increase in siderophoregenesis when pH values increased from 5.8 to 6.9, obtaining a maximum yield at neutral pH (6.9).

Figure 2a shows the siderophores production in a batch culture in M9S, 25 °C, pH 6.9. Although the molecular structure of the compound was not known at this stage, green-yellow chromophores were visualized under these culture conditions (Fig. 2b). Absorption occured at the same wavelength of pyoverdine like siderophores produced by *P. fluorescens* and *P. aeruginosa* (Kumar and Dube 1991; Meyer and Abdallah 1978). Therefore, siderophore production of *P. veronii* 2E could be monitored by measuring absorbance at 404 nm. The metabolite production started after an incubation of 6 h declining after 36 h. Siderophoregenesis was maximal at stationary phase of growth.

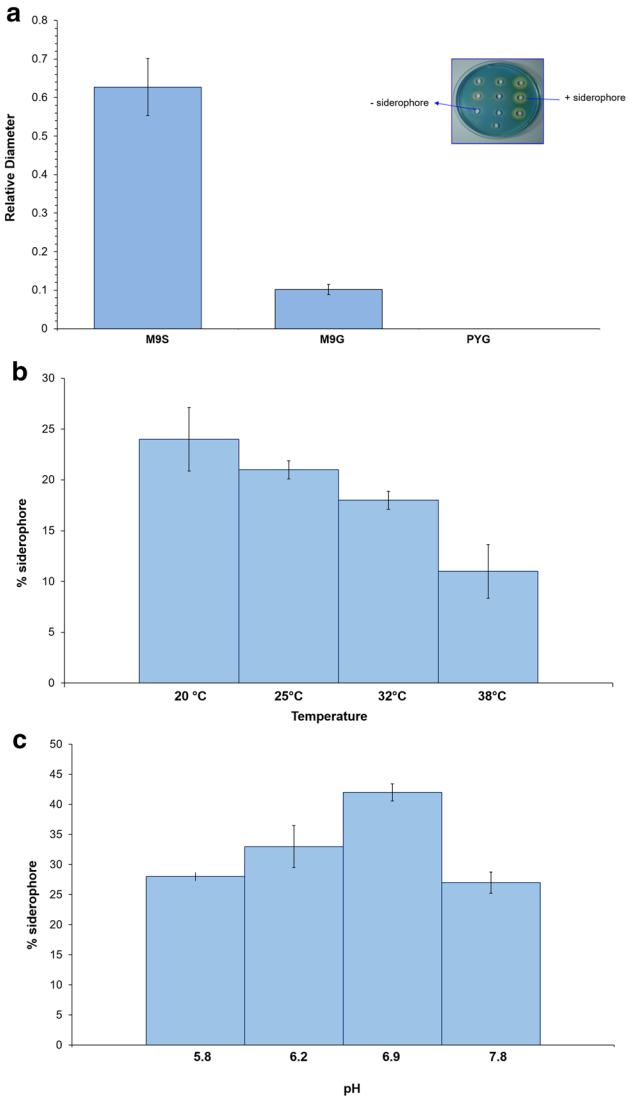

Fig. 1 **a** Siderophore production by *P. veronii* 2E in different culture media (M9S, M9G and PYG). Relative diameter is expressed as $(\text{diameter}/OD_{600nm})_{P.veronii\ 2E}/(\text{diameter}/OD_{600nm})_{P.aeruginosa\ PA01}$. Inserted image shows CAS-agar plates: +siderophore, presence of siderophore; -siderophore, absence of siderophore. **b** Effect of temperature (20 °C, 25 °C, 32 °C and 38 °C) on siderophore production. **c** Effect of pH (5.8, 6.2, 6.9 and 7.8) on siderophore production. Production is expressed as % siderophore, $[(Ar - As)/(Ar \times OD_{600nm})] \times 100$

Chemical characterization of molecules

Catechol and hydroxamate groups were detected with colorimetric reactions using the tests developed by Arnow (1973), Rioux et al. (1983) and Csáky (1948) respectively. As shown in Table 1, *P. veronii* 2E secreted siderophores with both hydroxamate and catechol groups. *P. aeruginosa* was used as positive control because it is a confirmed pyoverdine secretor in minimal medium, a siderophore with catechol, and hydroxamate groups. 2, 3-DHBA tested negative in Csáky assay and positive for Rioux and Arnow assay.

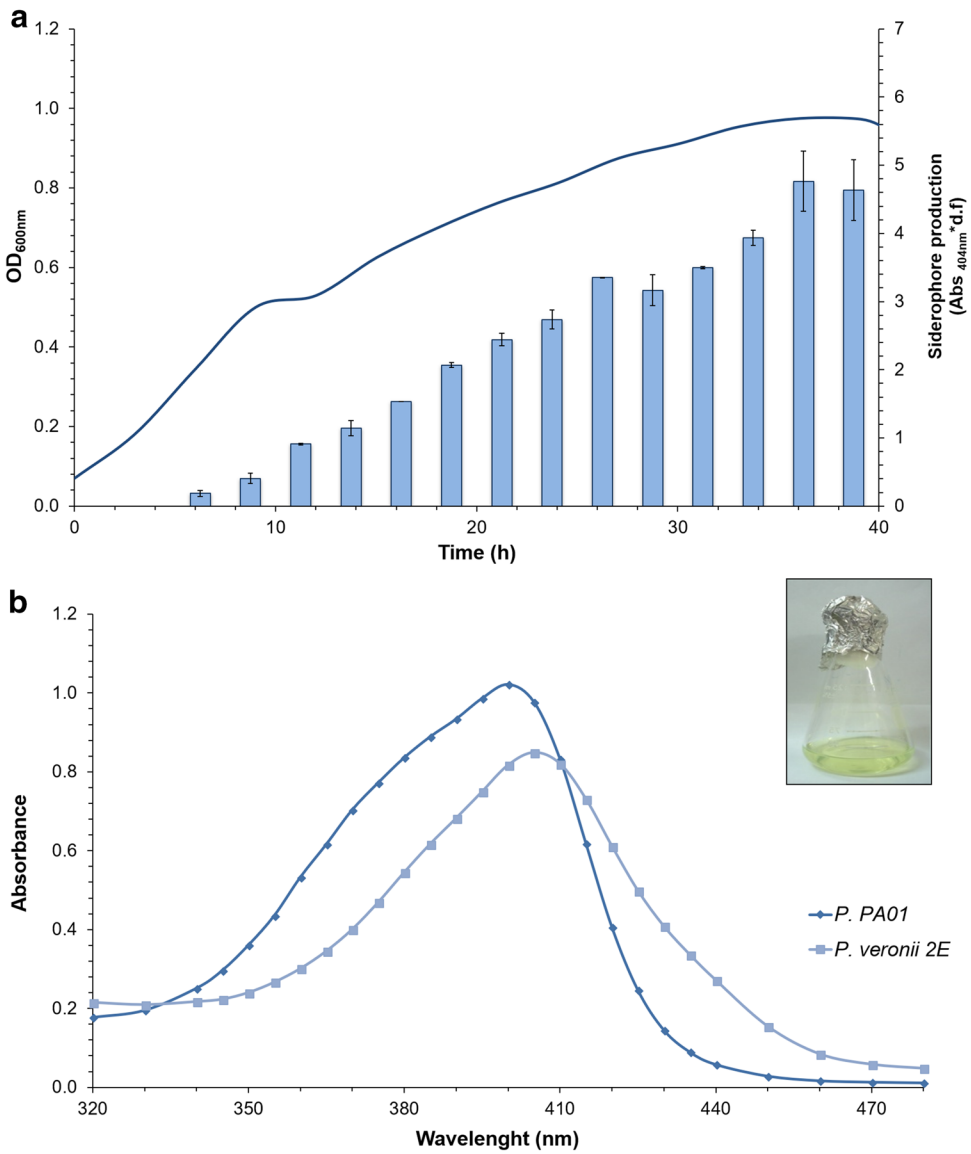

Fig. 2 **a** Siderophore production in time, expressed as production of siderophore (Absorbance supernatant $\lambda = 404$ nm × d.f, where d.f is the dilution factor) (Bars); growth curve by *P. veronii* 2E (solid line). **b** Absorbance spectrum of the supernatant of *P. aeruginosa* PA01 and *P. veronii* 2E; *P. veronii* 2E culture in M9S (inserted image in the upper right corner)

Table 1 Chemical characterization of siderophores secreted by *P. veronii* 2E

Bacteria/positive control	CAS	Hydroxamate Csáky assay	Catechol Arnow assay	Catechol Rioux assay
P. veronii 2E	++	++	+[b]	++
P. aeruginosa PA01	+++	+++	+[b]	++
2,3-DHBA	+	–	++[a]	+++

Assay scoring: strong reaction (+++), moderate (++), slight (+), no reaction (–)

[a]Red color formation

[b]Yellow color for supernatant from *P. veronii* 2E and *P. aeruginosa* PA01

Effect of metals on siderophore biosynthesis assays

In order to determine the influence of different metals on siderophore biosynthesis, *P. veronii* 2E was grown in M9S with different concentrations of Cd(II), Cu(II), Cr(III), Ni(II) and Zn(II). Results were expressed as (maximum absorbance) × $(OD_{600nm})^{-1}$, to evaluate siderophore production independently from the bacterial growth and to be able to compare between the different metals.

The presence of Zn(II) in M9S promoted the stimulation of metabolite production at all tested concentrations after 40 h of growth, and Zn(II) was the metal that induced

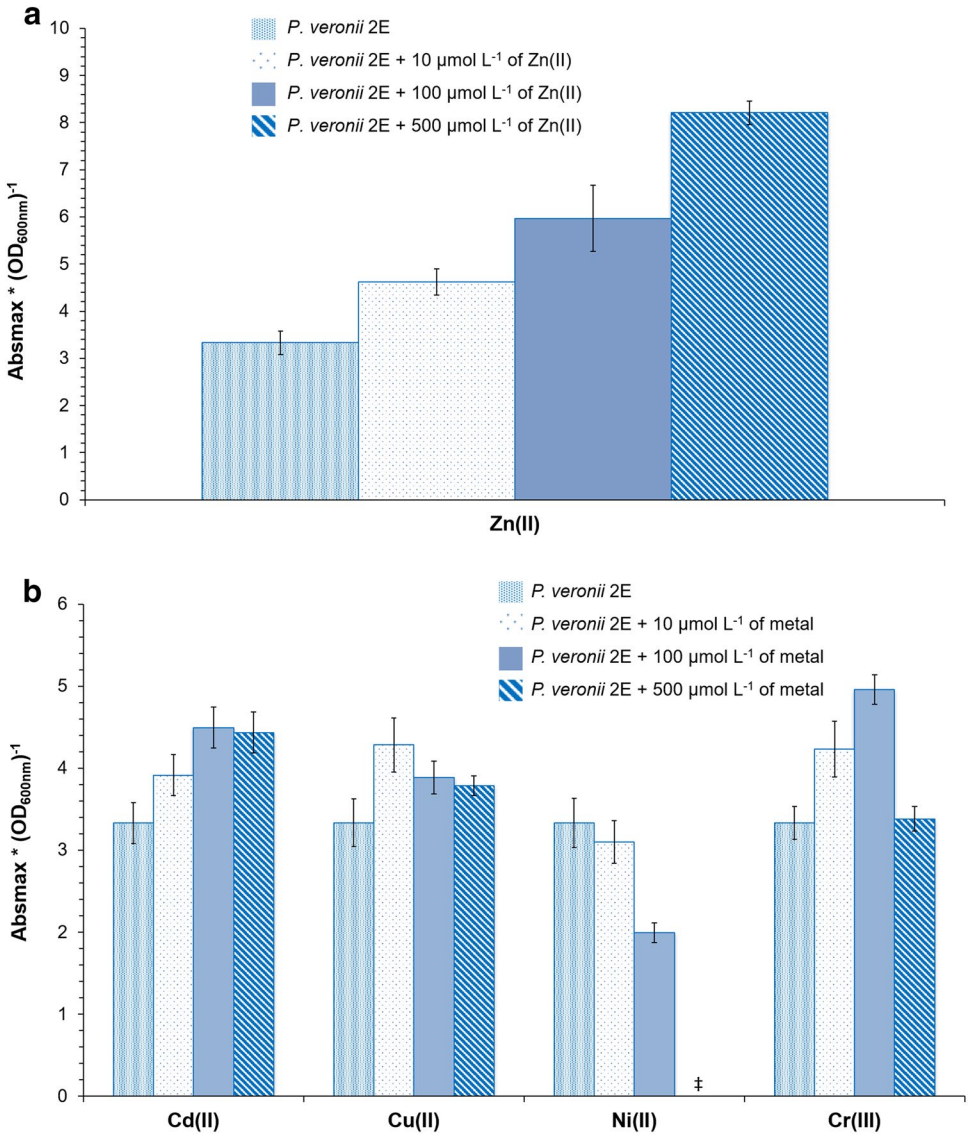

Fig. 3 Effect of dose–response of **a** Zn(II), **b** Cd(II), Cu(II), Ni(II) and Cr(III) on siderophore production. *P. veronii* 2E growth in M9S supplemented with 10, 100 and 500 µmol L^{-1} of metals. ‡Indicates no siderophore detection

siderophoregenesis the most (Fig. 3a). For the other metals studied, results are resumed in Fig. 3b: an increase in siderophore production was observed for a final concentration of 10 µmol L^{-1} and 100 µmol L^{-1} of Cd(II), Cr(III) and Cu(II). A decrease in secretion was observed with 500 µmol L^{-1} Cr(III) and no differences with the control were observed for Cd(II) and Cu(II). No stimulation was registered when Ni(II) concentration was 500 µmol L^{-1}.

Screening of metal–siderophore interactions

Siderophores secreted by *P. veronii* 2E contained functional groups such as catechol and hydroxamate that could interact with metals besides Fe(III). Supernatants from M9S cultures were supplemented with 1 µmol L^{-1}, 10 µmol L^{-1} and 100 µmol L^{-1} Cd(II), Cu(II), Cr(III), Ni(II) and Zn(II). The interaction was analyzed by spectrophotometry. All the metals studied, in at least one concentration, showed changes in peak shape and peak position in the absorption spectra, confirming a metal-siderophore interaction (Online Resource 2). For spectra obtained from Cd(II), Cu(II) and Zn(II) only when the final concentration was 100 µmol L^{-1}, the curves showed a shift in the maximum absorbance and a different shape in the case of Cd(II). For Ni(II) and Cr(III), these changes were observed from 10 µmol L^{-1} onwards, being more evident in the case of Ni(II).

Siderophores as Cd(II) ligand

The ASV technique was used to evaluate complexing capacity towards Cd(II) by a titration curve (Online Resource 3-a). The first part of this curve corresponded to the complexation equilibrium originated in the interaction between Cd(II) and the siderophores while the linear zone corresponded to

Cd(II) excess with respect to the binding sites. Cadmium hydroxide precipitation was not observed, which was also an evidence of the complexation with the organic ligands.

The analysis of the titration curves by Ružić and Scatchard linearizations allowed the determination of the number of ligands families and the conditional stability constant K′ of the metal complexes. In the Ružić graph (Online Resource 3-c), a good linear correlation was obtained with a single slope, showing that 1:1 ligand–metal approach is correct. The conditional stability constant obtained from Scatchard plot (Online Resource 3-b) was consistent with one family of moderate ligands (Log K′ 6.1 ± 0.1).

The ability of siderophores to remove and hence mobilize Cd(II) from diatomite is a relevant question from an environmental point of view. Diatomite is well known for metal retention and also very attractive due to low cost (Danil de Namor et al. 2012; Ramirez et al. 2017). For this purpose, the conditional stability constants of Cd(II) interaction for both materials were compared. The conditional stability constants were $(5.0 \pm 0.9) \times 10^3$ and $(1.2 \pm 0.3) \times 10^6$ for diatomite-Cd(II) and siderophore-Cd(II) interaction, respectively. This result suggests a stronger siderophore-Cd(II) interaction. Thus siderophores may be used as a useful tool for Cd(II) recovery to avoid final confinement of the immobilised metal in diatomite allowing further recycling.

Siderophore structural analysis

Mass spectrometry provided structural evidence of the presence of pyoverdine chromophores and the molecular weight of the unknown compound. The purification and molecular mass analysis were performed in the siderophore-Fe(III) fraction in order to reduce possible issues from the interaction between siderophores with Fe(III) traces. The nanoHPLC chromatogram of the siderophore-Fe(III) complexes was obtained (Online Resource 4), showing the different retention times strictly related to differences in molecular mass that can only arise from different chromophores and/or different side chains (Fuchs et al. 2001; Ye et al. 2013). The molecular mass of siderophore-Fe(III) was obtained from their doubly protonated molecular ions $[M + 2H^+]$ by ESI-MS. The results are given in Table 2 and the ESI mass spectrum of the peak at retention time 9.96 min is shown in Online Resource 5. Molecular ion peak at m/z 410.1 from 1242.4 u MS/MS (retention time 9.96 min) (Online Resource 5-a) represent the Fe-chromophore-side chain of purified siderophore-Fe, where the chromophore represent: 5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2-a]-quinolone-1-carboxylic acid and the side chain a succinic amide. Fragmented ion peaks obtained could be corresponded the next peptidic moiety: OHOrn = hydroxyornithine; Thr: threonine; Asn: asparagine; Ser: serine; FoOHOrn; formyl-hydroxyornithine; cOHOrn: cyclo-hydroxyornithine (Online Resource 5-b).

Table 2 Molecular masses and retention time of purified siderophore-Fe(III) complexes from *P. veronii* 2E

m/z Z = 2	Molecular mass (u)	Retention time (min)
613.2	1224.4	3.48
622.2	1242.5	3.84
599.2	1197.4	8.78
607.7	1213.4	8.87
598.7	1195.4	9.29
621.7	1242.4	9.96

u is atomic mass unit

Discussion

Pseudomonas veronii 2E was screened for siderophore production by CAS assay of Schwyn and Neilands (1987) with modifications. In the original assay, bacteria grew in the agar with CTAB, but since CTAB was toxic to *P. veronii* 2E, changes to universal CAS assay were performed. The modification consisted in using the supernatant from culture media, a lower concentration of buffer PIPES and avoiding the use of a carbon source (sterile conditions were not necessary). The experiment is based on a competition for Fe(III) established between the siderophores and the indicator dye CAS-CTAB: when siderophores remove Fe(III) from CAS-CTAB-Fe(III) complex, the release of the free dye is accompanied by a colour change (from blue to yellow-orange) (Inserted image in Fig. 1a).

The siderophore production in M9S (25 °C, pH 6.9) was detected after 6 h of incubation in batch and continued during the exponential phase achieving a maximum in stationary phase. Clearly, siderophore production was related to bacterial growth. *P. veronii* 2E usually grows in complex and mineral types of culture media, so siderophoregenesis was tested under both nutritional conditions. Siderophore production was detected in minimal media M9S and M9G but not in the complex medium PYG, in contrast to *P. aeruginosa* which secreted siderophores despite the medium composition. These results for *P. veronii* 2E may be associated with iron availability in rich media, being the most important factor in siderophore production. The optimal production was in M9S, suggesting that succinate as carbon source was efficiently used in siderophoregenesis by *P. veronii* 2E. Succinate is possibly related to the chemical structure of pyoverdines, in which acyl side chain on the chromophore comes from the acyl group of the succinate structure (Budzikiewiez 1993; Demange et al. 1987; Fuchs et al. 2001; Mehri et al. 2012). The secreted metabolite had a green–yellow colour

that absorbed at 404 nm, suggesting that the siderophores belonged to the pyoverdine family.

To improve the production conditions, secretion of the metabolite was studied at different pH values. The concentration of soluble Fe(III) and thus the production of siderophores, was affected by pH of the culture media. It was observed for other species belonging to the *Pseudomonas* genus that the Fe bioavailabilty determines the siderophore biosynthesis. Butaité et al. (2018), stated that bacteria gradually downscale siderophore production while iron bioavailability increased, that is under low pH (pH < 5.8) or weak chelators presence. Consequent with this, for *P. veronii* 2E, siderophore secretion was maximal at pH 6.9. However, with the lowest concentration of soluble Fe(III), observed at pH 7.8 (Online Resource 1), the maximum percentages of stimulation of siderophoregenesis were also expected. In contrast, the siderophore production was comparable to that obtained at pH 5.8. Meyer and Abdallah (1978) observed a degradation of 30% of siderophores from the pyoverdine group at pH higher than 8. Albrecht-Gary et al. (1994) reported the oxidation and degradation of the catechol group in pyoverdines in basic solutions. According to these evidences, molecule degradation at pH 7.8 should not be disregarded.

The siderophore secretion was clearly increased at low temperatures, being 25 °C the chosen condition for the siderophore production. The effect of temperature was also detected in other bacteria as *P. aeruginosa, Vibrio salmonicida* and *Salmonella Typhimurium* (Colquhoun and Sørum 2001; Sulochana et al. 2014; Worsham and Konisky 1984). In all cases siderophore production was enhanced at suboptimal temperatures, attributed to different phenomena which should not be disregarded in this case: the special activation of Fe-siderophore complex uptake, the increase in bacterial virulence or the evasion from bacterial competitors for Fe availability.

Although siderophores are secreted by a variety of bacteria, the chemical groups that coordinate Fe(III) are evolutionarily conserved. Most of these molecules possess catechol groups or/and hydroxamate which are involved in the interaction with iron. CAS assay only indicates the presence of siderophores, but this test does not provide structural information. Therefore, additional assays were performed to test the presence of catechol and hydroxamate groups. Pyoverdines contain in their structure a chromophore that gives them the characteristic green-yellow color. The biochemical assays performed (Csáky, Rioux and Arnow) allowed the detection of hydroxamate and catechol groups. Although the Arnow test is widely used when analyzing the presence of catechol motive in siderophores (Cornelis and Matthijs 2007; Paine 1994; Tortora et al. 2011), the characteristic red color of catechol group reactions is developed when the vicinal diols do not have substituents at positions 3 and 4. In that situation, the intermediate product is immediately stabilized by the addition of NaOH (Barnum 1977; Paine 1994; Rioux et al. 1983; Srideri et al. 2008). For this reason, the reaction is clearly positive for siderophores such as enterobactin (produced by *Escherichia coli*, Raymond et al. 2003) or vibriobactin (produced by *Vibrio cholerae*, Miethke and Marahiel 2007) corresponding to 2, 3-DHBA derivatives. For siderophores with positions 3 and 4 replaced, as pyoverdine group, the final product is an unstable yellow compound. For this type of compounds, the reaction is not complete and hence does not end in the red compound (Daveu et al. 1997; Rioux et al. 1983). In this case, the development of a yellow color was taken as positive, thus concluding that the catechol group present in *P. veronii* 2E siderophores had substituents in positions 3 and 4, consistent with the diol groups in pyoverdine chromophores. After this assay, the Rioux assay was shown to be positive for the siderophores produced by *P. veronii* 2E, which is more conclusive in the detection of catechol groups than Arnow test (Rioux et al. 1983; Velasquez et al. 2011). The results suggested that pyoverdine produced by *P. veronii* 2E had hydroxamate and catechol groups in their structure, involved in the complexation with Fe(III) (Miethke and Marahiel 2007; Neilands 1995; Saha et al. 2016). This preliminary detection was an advance in the elucidation of the structure. The mass spectra analysis of the siderophore-Fe(III) sample gave a molecular mass of approximately 1200 u. Mass spectrometry MS/MS provided structural evidence of the presence of chromophore: chr(5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2-a]-quinolone-1-carboxylic acid); and a dicarboxylic side chain succinic amide. These results revealed that the produced siderophores belonged to the family of pyoverdines, unreported up to now. This proposed structure contains cOHOrn as C-terminal aminoacid of the peptidic moiety, evidencing that this is a non-described siderophore chemically compatible to Class II pyoverdines classification (Meyer et al. 1997). NMR experiments using 500 MHz in combination with bioinformatic analyses will be needed to confirm the peptidic chain structure.

On the other hand, the secretion of siderophores could be affected by other metals (Braud et al. 2009a; Mehri et al. 2012; Schalk et al. 2011). In the case of Gram-negative bacteria, it is known that metal ions enter the cell mainly by diffusion across porins of the outer membrane into periplasm (Lutkenhaus 1977). The metal-siderophore interactions could affect the cation concentrations producing a decrease in their intracellular diffusion (Braud et al. 2010; Visca et al. 1992, 2002). The results showed that *P. veronii* 2E was capable of producing siderophores in absence of Fe(III) and in presence of 10 μmol L^{-1} of Zn(II), Cu(II), Cd(II) and Cr(III). Visca et al. (1992) observed that pyoverdine production by *Pseudomonas aeruginosa* was not affected by Zn(II), Ni(II) and Cu(II) at concentrations below 10 μmol L^{-1}; and Braud et al. (2009a) reported an induction of pyoverdine

secretion at 10 µmol L^{-1} Cu(II) and Ni(II). *P. veronii* 2E could secrete siderophores in the presence of metals as a strategy to decrease their toxic effects. Being Cd(II) the most harmful of the evaluated metals, it did not evidence the maximal stimulation of siderophoregenesis during bacterial growth. This surprising result could be understood in terms of the multiple detoxification mechanisms shown by *Pseudomonas veronii* 2E: biosorption by bacterial cells, interaction in biofilm matrix and complexation by extracellular polymeric substances, EPS (Daniel et al. 2016; Ferreira et al. 2017; Méndez et al. 2011; Vullo et al. 2008). In addition, in the assays performed in presence of Ni(II) and Cu(II), bacterial aggregates and biofilm formation were observed; and production of soluble EPS in presence of Cd(II) was registered. These are clear evidence that *P. veronii* 2E siderophore production is not the only strategy for metal detoxification. It had different alternatives for reducing harmful metal effects depending on the nature of the metal and its concentration. When the interaction between siderophores and metal cations other than iron was studied, the absorption spectra proved that pyoverdines produced by *P. veronii* 2E were able to interact with Zn(II), Cu(II), Cr(III), Cd(II) and Ni(II).

Being similar to zinc in chemistry, cadmium is associated in nature with zinc, exhibiting the highest toxicity. Cd emissions are related to zinc production as a by-product from zinc smelting and also to the production of Ni-Cd batteries, pigments, alloys, stabilizers for polyvinyl chloride (PVC) for example. As consequence, Cd-containing residues are of environmental concern because of the proved toxic effects on biota. So, Cd removal applying complexing agents would be an ecofriendly methodology for potential future metal recovery. In this way, Cd(II)-siderophore complexes were studied using electrochemical monitored titration (ASV technique). The results were consistent with one family of moderate ligands (Log K′ 6.1 ± 0.1). Values of pyoverdine affinity constants (Kf) with different metals were reported by different researchers, however the comparison is not possible as there are no conditional stability constants (K′) informed as in this work (Albrecht-Gary et al. 1994; Cézard et al. 2015; Chen et al. 1994; Cornu et al. 2014). According to Nieboer and Richardson (1980) and Duffus (2002) Fe(III) is a "hard" Lewis acid and strongly interacts with "hard" ligands, like oxygen, present in the catechol and hydroxamate groups; Cd(II), like Fe(II), is a borderline Lewis acid that interacts more weakly with these ligands.

Siderophores are effective in solubilizing and mobilizing different metals (Schalk et al. 2011) and even metalloids as arsenic. In fact, Nair et al. (2007) showed mobilization of arsenic from contaminated soil using siderophores from *P. azotoformans*. Edberg et al. (2010) indicated Ni and Co release from ore samples related to siderophore production by *P. fluorescens*. In this work, extraction of Cd(II) from diatomite was considered. These results suggested that the siderophores secreted by *P. veronii* 2E may be used as an efficient extracting agent for Cd(II) from adsorbent matrices as diatomite.

In the last decade, different roles and applications of siderophores in the environmental field have been investigated. Microbial siderophores contribute with Fe to plant nutrition enhancing growth under stress conditions and have been suggested as an alternative to pesticides by protecting them from pathogens. As consequence siderophore producers can be classified as *plant growth promoting bacteria* (PGPB). On the other hand, siderophore-assisted phytoextraction has received a particular interest (Ahmed and Holmstrom 2014; Rajkumar et al. 2010; Saha et al. 2016). Braud et al. (2009b) reported an increase of Cr and Pb accumulation in maize shoots when a contaminated soil was inoculated with the siderophore-producing bacteria *P. aeruginosa*. Dimkpa et al. (2009) showed that hydroxamate siderophores secreted by *Streptomyces tendae* F4 promoted plant growth and enhanced uptake of Cd and Fe by sunflowers. Further research will involve studying the potential role of the siderophores produced by *P. veronii* 2E in metal availability for plants as the potential use as PGPB.

Conclusions

This study revealed that *P. veronii* 2E secreted unreported Class II pyoverdine-siderophores able to efficiently complex metal cations. In addition, ASV study of siderophore-Cd(II) interactions showed the presence of a moderate single family of ligands with a potential use in Cd(II) removal and hence subsequent metal recovery. Therefore, the obtained results indicate that the siderophores produced by the non-pathogenic metal resistant bacterium *P. veronii* 2E are promising in bioremediation applications.

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References

Ahmed E, Holmstrom SJM (2014) Siderophores in environmental research: roles and applications: minireview. Microb Biotechnol 7:196–208. https://doi.org/10.1111/1751-7915.12117

Albrecht-Gary A-M, Rochel SN, Ocaktan AZ, Abdallah MA (1994) Bacterial iron transport: coordination properties of pyoverdine PaA, a peptidic siderophore of *Pseudomonas aeruginosa*. Inorg Chem 33:6391–6402. https://doi.org/10.1021/ic00104a059

Alessandrello M, Vullo DL (2016) Economical fermentation media for the production of a whole cell catalyst for the treatment of Cr(VI)-containing wastewaters. Rev Argent Microbiol 48:245–251

Arnow LE (1973) Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J Biol Chem 118:531–537

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci USA 102:11076–11081. https://doi.org/10.1073/pnas.0504266102

Barnum DW (1977) Spectrophotometric determination of catechol, epinephrine, DOPA, dopamine and other aromatic vic-diols. Anal Chim Acta 89:157–166. https://doi.org/10.1016/S0003-2670(01)83081-6

Barrero JM, Moreno-Bondi MC, Pérez-Conde MC, Cámara C (1993) A biosensor for ferric ion. Talanta 40:1619–1623

Boukhalfa H, Crumbliss AL (2002) Chemical aspects of siderophore mediated iron transport. Biometals 15:325–339

Braud A, Hoegy F, Jezequel K, Lebeau T, Schalk IJ (2009a) New insights into the metals specificity of the *Pesudomonas aeruginosa* pyoverdine-iron uptake pathway. Environ Microbiol 11:1079–1091. https://doi.org/10.1111/j.1462-2920.2008.01838.x

Braud A, Jézéquel K, Bazot S, Lebeau T (2009b) Enhanced phytoextraction of an agricultural Cr-Hg- and Pb-contaminated soil by bioaugmentation with siderophore producing bacteria. Chemosphere 74:280–286. https://doi.org/10.1016/j.chemosphere.2008.09.013

Braud A, Geoffroy V, Hoegy F, Mislin GL, Schalk IJ (2010) Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal accumulation in *Pseudomonas aeruginosa* and increases bacterial metal tolerance. Environ Microbiol Rep 2:419–425. https://doi.org/10.1111/j.1758-2229.2009.00126.x

Braun V, Pramanik A, Gwinner T, Köberle M, Bohn E (2009) Sideromycins: tools and antibiotics. Biometals 22:3–13. https://doi.org/10.1007/s10534-008-9199-7

Budzikiewiez H (1993) Secondary metabolites from fluorescent pseudomonads. FEMS Microbiol Rev 10:209–228

Butaitė E, Kramer J, Wyder S, Kümmerli R (2018) Environmental determinants of pyoverdine production, exploitation and competition in natural *Pseudomonas* communities. Environ Microbiol. https://doi.org/10.1111/1462-2920.14355

Cézard C, Farvacques N, Sonnet P (2015) Chemistry and biology of pyoverdines, *Pseudomonas* primary siderophores. Curr Med Chem 22:165–186

Chen Y, Jurkevitch E, Bar-Ness E, Hadar Y (1994) Stability constants of pseudobactin complexes with transition metals. Soil Sci Soc Am J 58:390–396

Colquhoun DJ, Sørum H (2001) Temperature dependent siderophore production in *Vibrio salmonicida*. Microb Pathog 31:213–219. https://doi.org/10.1006/mpat.2001.0464

Cornelis P, Matthijs S (2007) *Pseudomonas* siderophores and their biological significance. In: Varma A, Chincholkar SB (eds) Microbial siderophore. Springer, Berlin, pp 121–133

Cornu JY, Elhabiri M, Ferret C, Geoffroy VA, Jezequel K, Leva Y, Lollier M, Schalk IJ, Lebeau T (2014) Contrasting effects of pyoverdine on the phytoextraction of Cu and Cd in a calcareous soil. Chemosphere 103:212–219. https://doi.org/10.1016/j.chemosphere.2013.11.070

Cox CD, Graham R (1979) Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. J Bacteriol 37:1357–1364

Crowley D (2006) Microbial siderophores in plant rhizosphere. In: Barton L, Abadia J (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer, Netherlands, pp 169–189

Csáky TZ (1948) On the estimation of bound hydroxylamine in biological materials. Acta Chem Scand 2:450–454. https://doi.org/10.3891/acta.chem.scand.025-0450

Daniel M, Barrionuevo M, Doyle S, Vullo DL (2016) Kinetics of *Pseudomonas veronii* 2E biofilm development under. Biochem Eng J 105:150–158. https://doi.org/10.1016/j.bej.2015.09.001

Danil de Namor AF, El Gamouz A, Frangie S, Martinez V, Valiente L, Webb OA (2012) Turning the volume down on heavy metals using tuned diatomite. A review of diatomite and modified diatomite for the extraction of heavy metals from water. J Hazard Mater 241–242:14–31

Daveu C, Servy C, Dendane M, Marin P, Ducrocq C (1997) Oxidation and nitration of catecholamines by nitrogen oxides derived from nitric oxide. Nitric Oxide Biol Ch 1:234–243. https://doi.org/10.1006/niox.1997.0123

Demange P, Wendenbaum S, Bateman A, Dell A, Meyer JM, Abdallah MA (1987) Bacterial siderophores: structure and physicochemical properties of pyoverdins and related compounds. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. VCH, Weinkeim, pp 167–187

Dimkpa C, Merten D, Svatos A, Büchel G, Kothe E (2009) Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. J Appl Microbiol 107:1687–1696. https://doi.org/10.1111/j.1365-2672.2009.04355.x

Duffus JH (2002) "Heavy metals"-A meaningless term? Pure Appl Chem 74:793–807

Edberg F, Kalinowski BE, Holmström SJM, Holm K (2010) Mobilization of metals from uranium mine waste: the role of pyoverdines produced by *Pseudomonas fluorescens*. Geobiology 8:278–292. https://doi.org/10.1111/j.1472-4669.2010.00241.x

Ferreira ML, Casabuono AC, Stacchiotti ST, Couto AS, Ramirez SA, Vullo DL (2017) Chemical characterization of *Pseudomonas veronii* 2E soluble exopolymer as Cd(II) ligand for the biotreatment of electroplating wastes. Int Biodeterior Biodegrad 119:605–613. https://doi.org/10.1016/j.ibiod.2016.10.013

Fuchs R, Schäfer M, Geoffroy V, Meyer JM (2001) Siderotyping-a powerful tool for the characterization of pyoverdines. Curr Top Med Chem 1:31–57. https://doi.org/10.2174/1568026013395542

Garavaglia L, Cerdeira SB, Vullo DL (2010) Chromium (VI) biotransformation by beta- and gamma-proteobacteria from natural polluted environments: a combined biological and chemical treatment for industrial wastes. J Hazard Mater 15:104–110. https://doi.org/10.1016/j.jhazmat.2009.09.134

Gillam AH, Lewis AG, Andersen RJ (1981) Quantitative determination of hydroxamic acids. Anal Chem 53:841–844. https://doi.org/10.1021/ac00229a023

Guan LL, Kanoh K, Kamino K (2001) Effect of exogenous siderophores on iron uptake activity of marine bacteria under iron-limited conditions. Appl Environ Microbiol 4:1710–1717. https://doi.org/10.1128/AEM.67.4.1710-1717.2001

Huang Y, Jiang Y, Wang H, Wang J, Shin MC, Byun Y, He H, Liang Y, Yang VC (2013) Curb challenges of the "Trojan Horse" approach: smart strategies in achieving effective yet safe cell-penetrating peptide-based drug delivery. Adv Drug Deliv Rev 65:1299–1315. https://doi.org/10.1016/j.addr.2012.11.007

Johnstone T, Nolan E (2015) Beyond iron: non-classical biological functions of bacterial siderophores. Dalton Trans 44:6320–6339. https://doi.org/10.1039/C4DT03559C

Kumar B, Dube H (1991) Plant growth-promoting activity of fluorescent *Pseudomonas* from tomato rhizoplane. Indian J Exp Biol 29:366–370

Lutkenhaus JF (1977) Role of mayor outer membrane protein in *Escherichia coli*. J Bacteriol 131:631–637

Martell E, Smith R (2010) NIST standard reference database 46. NIST critically selected stability constants of metal complexes. Version 8.0. http://www.nist.gov/srd/nist46.cfm

Masalha J, Kosegarten H, Elmaci O, Mengel K (2000) The central role of microbial activity for iron acquisition in maize and sunflower. Biol Fertil Soils 30:433–439. https://doi.org/10.1007/s003740050

Mehri I, Khessairi A, Turki Y (2012) Effect of dose-response of zinc and manganese on siderophore production. Am J Environ Sci 8:143–151

Méndez N, Ramírez S, Ceretti H, Zalts A, Candal R, Vullo DL (2011) *Pseudomonas veronii* 2E surface interactions with Zn(II) and Cd(II). Global J Environ Sci Technol 1:3

Merja M, Tiina M-S (1994) Methods for detection of *Pseudomonas* siderophores. J Microbiol Methods 19:223–234

Meyer JM, Abdallah MA (1978) The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J Gen Microbiol 107:319–328. https://doi.org/10.1099/00221287-107-2-319

Meyer JM, Stintzi A, De Vos D, Cornelis P, Tappe R, Taraz K, Budzikiewicz H (1997) Use of siderophores to type pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. Microbiology 143:35–43. https://doi.org/10.1099/00221287-143-1-35

Meyer JM, Geoffroy VA, Baida N, Gardan L, Izard D, Lemanceau P, Achouak W, Palleroni NJ (2002) Siderophore typing, a powerful tool for the identification of fluorescent and nonfluorescent *Pseudomonas*. Appl Environ Microbiol 68:2745–2753. https://doi.org/10.1128/AEM.68.6.2745-2753.2002

Miethke M, Marahiel M (2007) Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev 7:413–451. https://doi.org/10.1128/MMBR.00012-07

Mollmann U, Heinisch L, Bauernfeind A, Kohler T, Ankel-Fuchs D (2009) Siderophores as drug delivery agents: application of the "Trojan Horse" strategy. Biometals 22:615–624. https://doi.org/10.1007/s10534-009-9219-2

Nair A, Juwarkar A, Singh S (2007) Production and characterization of siderophores and its applications in arsenic removal from contaminated soil. Water Air Soil Pollut 180:199–212

Neilands JB (1995) Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270:26723–26726

Nieboer E, Richardson D (1980) The replacement of the nondescript term heavy metals by a biologically and chemically significant classification of metal ions. Environ Pollut 1:3–26

Paine SM (1994) Detection, isolation, and characterization of siderophores. Methods Enzymol 235:329–344

Rajkumar M, Ae N, Prasad MN, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol 28:142–149. https://doi.org/10.1016/j.tibtech.2009.12.002

Ramirez SA, Zalts A, Fernández Morantes C, Torres Sánchez RM, Sosa GL (2017) Adsorción de Zn(II) en diatomitas modificadas con ligandos: estudios en equilibrio para su aplicación en el tratamiento de efluentes. In: Aspromonte S, Boix A, Bosko L, Gomez L (eds) Ambiente y desarrollo sostenible desde una perspectiva multidisciplinaria. Sociedad Argentina de Ciencia y Tecnologia Ambiental, Buenos Aires, pp 483–488

Raymond KN, Dertz EA, Kim SS (2003) Enterobactin: an archetype for microbial iron transport. Proc Natl Acad Sci USA 100:3584–3588. https://doi.org/10.1073/pnas.0630018100

Rioux C, Jordan DC, Rattray JB (1983) Colorimetric determination of catechol siderophores in microbial cultures. Anal Biochem 133:163–169

Ružić I (1996) Trace metal complexation at heterogeneous binding sites in aquatic systems. Mar Chem 53:1–15. https://doi.org/10.1016/0304-4203(96)00008-4

Saha R, Saha N, Donofrio RS, Bestervelt LL (2012) Microbial siderophores: a mini review. J Basic Microbiol 52:1–15. https://doi.org/10.1002/jobm.201100552

Saha M, Sarkar S, Sarkar B, Sharma BK, Bhattacharjee S, Tribedi P (2016) Microbial siderophores and their potential applications: a review. Environ Sci Pollut Res Int 23:3984–3999. https://doi.org/10.1007/s11356-015-4294-0

Sayyed RZ, Badgujar MD, Sonawane HM, Mhaske MM, Chincholkar SB (2005) Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads. Indian J Biotechnol 4:484–490

Scatchard G (1949) The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672. https://doi.org/10.1111/j.1749-6632.1949.tb27297.x

Schalk IJ, Hannauer M, Braud A (2011) New role for bacterial siderophores in metal transport and tolerance. Environ Microbiol 13:2844–2854. https://doi.org/10.1111/j.1462-2920.2011.02556.x

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Srideri M, Kumar KG, Mallaiah KV (2008) Production of catechol-type os siderophores by *Rhizobium sp*. isolated from stem nodules of *Sesbania procumbens* (Roxb.) W and A. Res J Microbiol 3:282–287. https://doi.org/10.3923/jm.2008.282.287

Sulochana MB, Jayachandra SY, Anil Kumar S, Parameshwar AB, Mohan Reddy K, Dayanand A (2014) Siderophore as a potential plant growth-promoting agent produced by *Pseudomonas aeruginosa* JAS-25. Appl Biochem Biotechnol 174:297–308. https://doi.org/10.1007/s12010-014-1039-3

Tortora ML, Diaz-Ricci JC, Pedraza RO (2011) *Azospirillum brasilense* siderophore with antifungal activity against *Colletotrichum acutatum*. Arch Microbiol 193:275–286. https://doi.org/10.1007/s00203-010-0672-7

Velasquez I, Nunn BL, Ibisanmi E, Goodlett DR, Hunter KA, Sander SG (2011) Detection of hydroxamate siderophores in coastal and Sub-Antartic waters off the South Eastern Coast of New Zeland. Mar Chem 126:97–107. https://doi.org/10.1016/j.marchem.2011.04.003

Visca P, Colotti G, Serino L, Verzili D, Orsi N, Chiancone E (1992) Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. Appl Environ Microbiol 58:2886–2893

Visca P, Leoni L, Wilson MJ, Lamont IL (2002) Iron transport and regulation, cell signaling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. Mol Microbiol 45:1177–1190

Visca P, Imperi F, Lamont IL (2007) Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol 15:22–30. https://doi.org/10.1016/j.tim.2006.11.004

Vullo DL, Ceretti HM, Daniel MA, Ramirez SA, Zalts A (2008) Cadmium, zinc and copper biosorption mediated by *Pseudomonas veronii* 2E. Bioresour Technol 99:5574–5581. https://doi.org/10.1016/j.biortech.2007.10.060

Worsham PL, Konisky J (1984) Effect of growth temperature on the acquisition of iron by *Salmonella typhimurium* and *Escherichia coli*. J Bacteriol 158:163–168

Ye L, Ballet S, Hildebrand F, Laus G, Guillemyn K, Raes J, Matthijs S, Martins J, Cornelis P (2013) A combinatorial approach to the structure elucidation of pyoverdine siderophore produced by a *Pseudomonas putida* isolate and the use of pyoverdine as a taxonomic marker for typing *P. putida* subspecies. Biometals 26:561–575. https://doi.org/10.1007/s10534-013-9653-z