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Detection and differentiation of microbial siderophores by isoelectric focusing and chrome azurol S overlay

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Siderophores are microbial, low molecular weight iron-chelating compounds. Fluorescent Pseudomonads produce different, strain-specific fluorescent siderophores (pyoverdines) as well as non-fluorescent siderophores in response to low iron conditions. We present an isoelectric focusing method applicable to unpurified as well as to purified pyoverdine samples where the fluorescent siderophores are visualized under UV illumination. Siderophores from different *Pseudomonas* sp., amongst which are *P. aeruginosa*, *P. fluorescens* and *P. putida*, including egg yolk, rhizospheric and clinical isolates as well as some derived Tn5 mutants were separated by this technique. Different patterns could be observed for strains known to produce different siderophores. The application of the chrome azurol S assay as a gel overlay further allows immediate detection of non-fluorescent siderophores or possibly degradation products with residual siderophore activity. The method was also applied to other microbial siderophores such as deferrioxamine B.

Keywords: CAS assay, isoelectric focusing, Pseudomonas, pyoverdine, siderophore

Introduction

Under iron-limiting conditions, fluorescent Pseudomonads (*P. aeruginosa*, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. syringae*, *P. tolaasii*) generally produce a yellow–green pigment (pyoverdine) which functions as a siderophore. The pyoverdine structure shows a fluorescent chromophore, which is a quinoline derivative, and a peptide arm of six to nine amino acid residues (reviewed in Höfte 1993). In the well characterized *P. aeruginosa* pyoverdine, the binding of iron is due to the presence of a catecholate group on the chromophore moiety and hydroxamate groups provided by δ-*N*-hydroxy ornithine derivatives (Tappe *et al.* 1993). Alternatively, in other fluorescent Pseudomonads, a hydroxy acid group from the residue of D-threo-β-hydroxy aspartic acid may participate in iron binding together with a hydroxamate group (Teintze *et al.* 1981). The peptide chain of pyoverdines is extremely variable amongst species and even amongst strains, both in number and type of residues, which results in specificity barriers in the uptake of

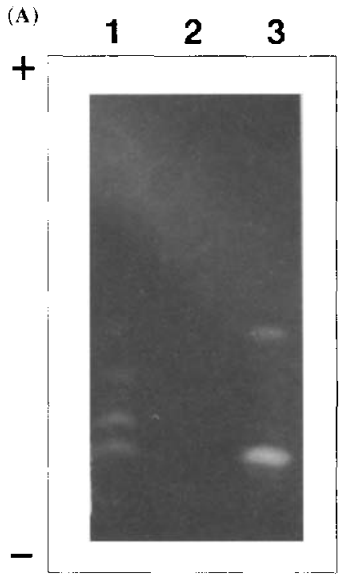

Figure 1. Patterns of microbial siderophores after IEF: (**A**) visualized under UV light and (**B**; see page 288) as detected in the CAS overlay. Wild-type *P. fluorescens* ATCC 17400 (lanes 3), pyoverdine-negative mutant 3G6 (lanes 2), fluorescent, pyoverdine-positive mutant 2G11 (lanes 1). Anode (+) and cathode (−).

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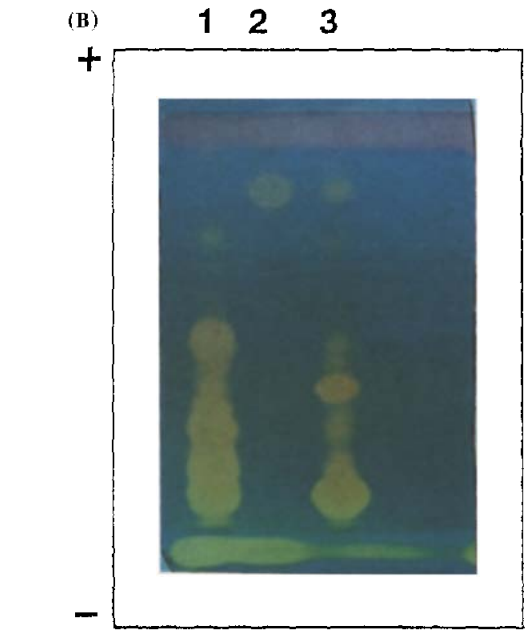

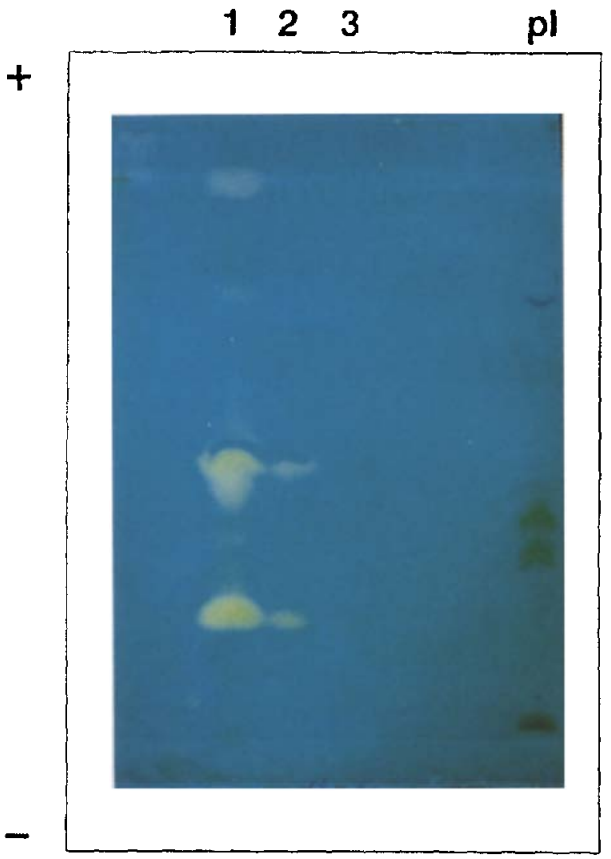

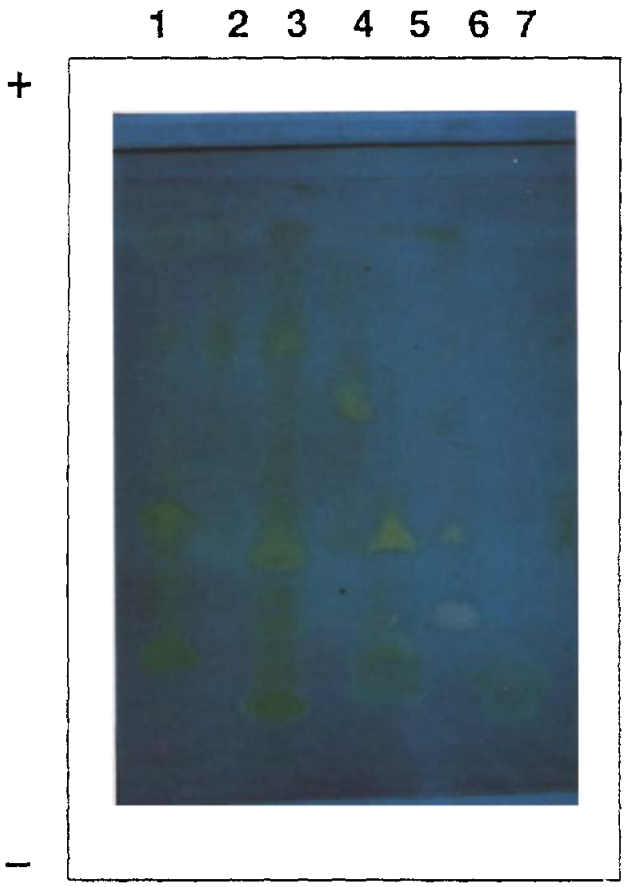

Figure 2. Detection of partially purified *P. aeruginosa* PA01 siderophores in the CAS overlay after IEF. Estimated total siderophore loading: lane 1, 1.76 nmole siderophores; lane 2, 0.176 nmole siderophores; lane 3, 0.0176 nmole siderophores; p*I*, p*I* standards. Anode (+) and cathode (−).

pyoverdines between different strains (Hohnadel & Meyer 1988), but which can also be exploited in electrophoretic differentiation. Until now, apart from biochemical analysis, differences in pyoverdines could only be investigated by cross-uptake assays of radioactively labeled ferrisiderophores, by immunological techniques or by cross-growth stimulation assays which are time consuming and not entirely reliable (Cornelis *et al.* 1989, Fekete 1993). Paper electrophoresis followed by siderophore detection through spraying with chrome azurol S (CAS) solution was described by Fekete (1993).

The potential for antagonism against phytopathogens like Gaeumannomyces, Pythium and Fusarium which is displayed by several fluorescent Pseudomonads (discussion in Leong 1986, Neilands & Leong 1986, Bakker *et al.*

Figure 3. Detection of partially or extensively purified microbial siderophores in the CAS overlay after IEF. Partially purified pyoverdine from *P. aeruginosa* PA01 (lane 1) and PA6 (lane 2), partially purified pyoverdine from *P. fluorescens* ATCC 17400 (lane 3), purified pseudobactins from *P. putida* WCS 358 (lane 4), from *Pseudomonas* sp. BN7 (lane 5) and from *Pseudomonas* sp. B10 (lane 6), and purified deferrioxamine B (lane 7). Anode (+) and cathode (−).

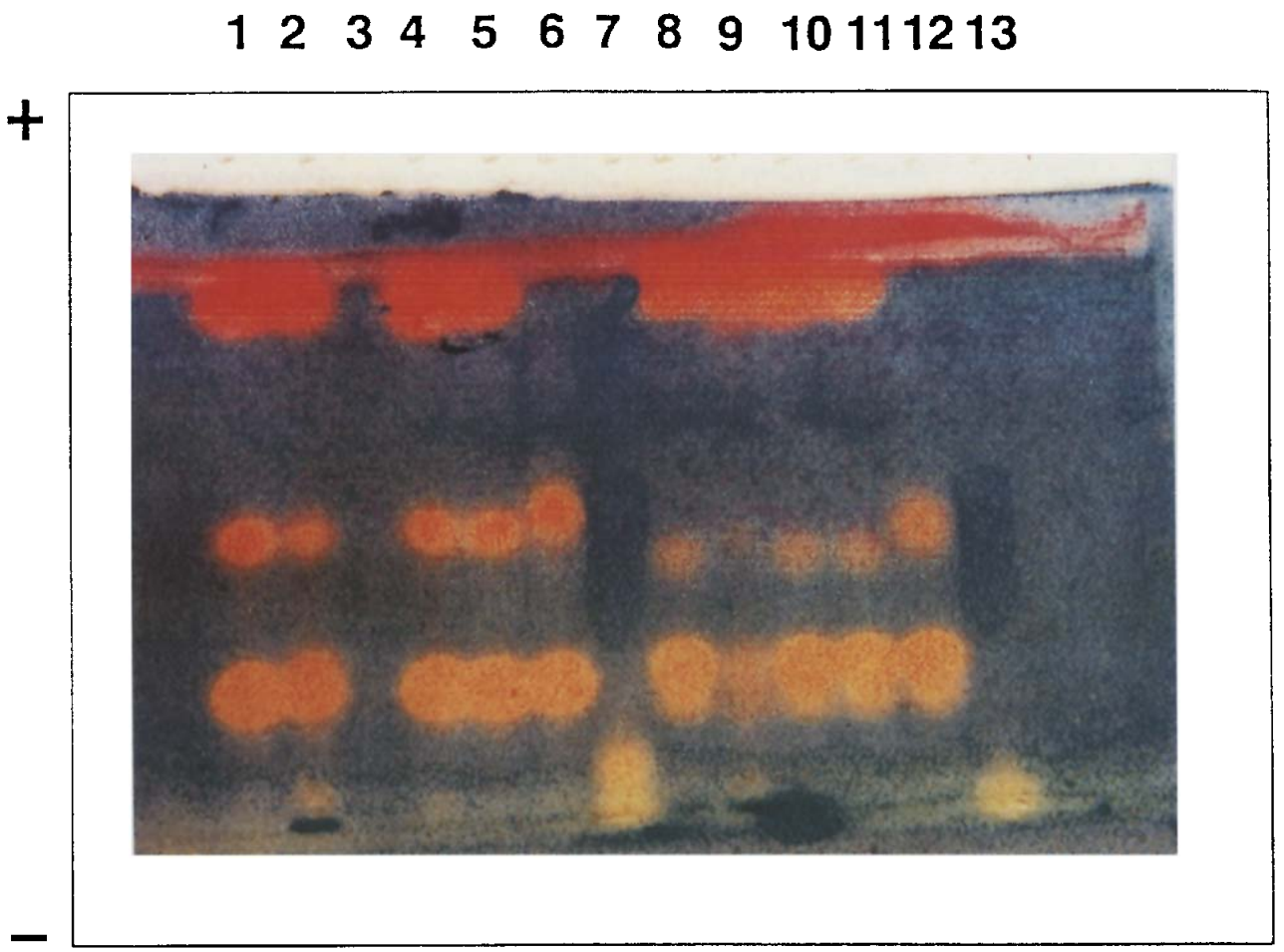

Figure 4. Patterns of partially purified siderophores after IEF as detected in the CAS overlay of *P. aeruginosa* cultures grown in the presence of different metal ions (CAA medium + filter-sterilized metal ion solutions). Lanes 1–7, PA01; lanes 8–13, PA3; lanes 1 and 8, no metal ions added; lanes 2 and 9, + 0.5 mM Fe; lanes 4 and 10, + 2 mM Zn; lanes 5 and 11, + 2 mM Cd, lanes 6 and 12, + 2 mM Ni; lanes 7 and 13, + 2 mM Al. Anode (+) and cathode (−). The figure shows a color scan of the original CAS overlay gel.

1993) is often attributed to pyoverdines, though this remains debatable in many cases, even if antagonism is iron regulated (e.g. Gill & Warren 1988). The screening of strains producing new pyoverdines and the study of siderophore diversity gains importance, but often requires purification and identification. Siderophores can be used as a marker for the identification of rhizosphere strains, of plant pathogenic *P. syringae* isolates or as an epidemiological marker for the human nosocomial pathogen *P. aeruginosa* (Cornelis *et al.* 1989). The method can also be used for screening mutants in siderophore biosynthesis or regulation (e.g. Höfte *et al.* 1993) or for following time-course experiments in siderophore production. More fundamental is the interest in the molecular aspects of iron uptake and iron sensing due to various types of siderophores.

We present an isoelectric focusing (IEF) method with subsequent siderophore detection by both fluorescence under UV illumination and by iron chelation through a CAS overlay. The technique allows a rapid and very reproducible assessment of siderophore diversity and of strain identity.

Materials and methods

Bacterial strains and culture media

P. fluorescens ATCC 17400 was originally isolated from egg yolk. Strain 3G6 is a pyoverdine-negative Tn5 mutant of *P. fluorescens* ATCC 17400 and strain 2G11 is a pyoverdine-positive Tn5 mutant from the same strain ATCC 17400, unable to grow in the presence of the strong iron chelator EDDHA (Cornelis *et al.* 1992). *P. aeruginosa* PA01 ATCC 15692 is a type strain, *P. aeruginosa* PA3 and PA6 are clinical isolates (UCL, Brussels), and *P. aeruginosa* 7NSK2 is a rhizosphere isolate (also see Höfte *et al.* 1993).

Bacteria were grown in casamino acid medium (CAA) for 48 h to get maximal production of pyoverdine (Höfte *et al.* 1993). *P. aeruginosa* strains were grown at 37 °C while the other fluorescent Pseudomonads were grown at 28 °C; cultures were shaken at 150 r.p.m. Metal ions were added as filter-sterilized solution to the autoclaved media with the final concentrations as indicated in Figure 4. The following salts were used for the solutions: $FeCl_3$, $ZnSO_4$, $CdCl_2$, $NiCl_2$ and $AlCl_3$.

Horizontal thin layer IEF and UV visualization of siderophores

This was performed with electrofocusing gels at pH 3.5–9.5 (Ampholine; Pharmacia, Brussels, Belgium) at 4 °C and 10 W for about 2 h, with a set of p*I* standards (p*I* 4.65–9.6 from BioRad Laboratories, Nazareth, Belgium). Volumes of 20–80 µl were applied. UV illumination (254 nm) immediately after the IEF run showed the fluorescent siderophore bands.

CAS overlay

This allows detection of siderophores, including non-fluorescent compounds. The overlay method was adapted from the CAS shuttle solution for siderophore assay in Schwyn & Neilands (1987). The CAS shuttle solution, as described by these authors, is made by adding to a 100 ml volumetric flask: 6 ml 10 mM (in water) hexadecyl trimethyl ammonium bromide (HDTMA), subsequently diluted to about 20 ml with water, 1.5 ml 1 mM (in 10 mM HCl) $FeCl_3$, 7.5 ml 2 mM CAS (Sigma, St Louis, MO, USA), which is added slowly to the previous volumes while stirring and 36.25 ml piperazine solution (pH 5.6; 4.307 g anhydrous piperazine in 30 ml water with 6.25 ml 12 M HCl added dropwise) and 10 ml of a 5-sulfosalicylic acid solution (in water) for a final concentration of 4 mM. The volume is adjusted to 100 ml. The solution is blue–violet and should be stored in the dark.

Agarose gels (0.8–1%, 1 mm thickness) containing the CAS shuttle solution were poured in a cassette of glass plates (pre-warmed at 50 °C) with a plastic sheet (GelBond film for agarose gels, LKB-Pharmacia, Uppsala, Sweden), hydrophilic side towards the agarose gel. The plastic sheet with the agarose gel was carefully overlayered on IEF gels shortly after the run. Yellow to colorless spots develop within minutes in the overlay if siderophores are present.

Overlay gels can be stored for several days after pouring, but preferably freshly poured overlays are used. The regular CAS solution as proposed by Schwyn & Neilands (1987) is also applicable: however, the CAS shuttle solution gave, in our experience, better results as to color contrast and to speed of signal development.

Partial purification of siderophores

This was performed by applying crude supernatant to a C_{18} column (2 × 0.5 cm) and eluting the yellow–green pigments in 50% methanol. The samples were then evaporated to dryness in a desiccator and redissolved in a minimal volume of distilled water. The samples were either applied directly to the IEF gel or kept at −20 °C. Pseudomonas total siderophore concentrations were estimated spectrophotometrically using an extinction coefficient $\varepsilon_{400} = 2 \times 10^4\ M^{-1}\ cm^{-1}$ (Meyer & Abdallah, 1978).

Purified siderophores

Pseudobactin of rhizospheric *Pseudomonas* sp. B10, BN7 and *P. putida* WCS358 and deferrioxamine B were kindly provided by Dr W. Bitter (Utrecht, the Netherlands).

Results and discussion

Visualization of siderophores

Figure 1 compares the pattern of siderophores obtained with a separation of crude culture supernatant from *P. fluorescens* ATCC 17400, the pyoverdine-negative mutant 3G6 and the pyoverdine-positive mutant 2G11. Several bands were observed under UV illumination for the wild-type ATCC 17400 (Figure 1A, lane 3) and the same major bands were observed after overlaying the IEF gel with an agarose gel containing the CAS solution (Figure 1B, lane 3). For the mutant 3G6, no band could by visualized under UV (Figure 1A, lane 2) but one siderophore, which is also present in the wild-type supernatant, could be detected by the CAS overlay (Figure 1B, lane 2).

From this result it is apparent that UV illumination allows a rapid screening but fails to show a number of compounds which do not or weakly fluoresce, yet behave as siderophores. The siderophore (transiently) produced by 3G6, which has an acidic p*I*, showed a very faint fluorescence under certain conditions but can easily be overlooked. This particular siderophore would also have been masked in a simple UV-Vis spectrophotometric assay since no particular absorption peak could be detected in the 3G6 supernatant. Recently, salicylic acid was reported as a *P. fluorescens* siderophore (Meyer *et al.* 1992). We have circumstantial evidence that the compound produced by strain 3G6 is a salicylate derivative (unpublished results).

Figure 2 shows the sensitivity of the CAS overlay technique. The three lanes were loaded with 1.76, 0.176 and 0.0176 nmole total PA01 pyoverdine solution, respectively (as estimated spectrophotometrically). The two major bands are still visible down to the lowest concentration using the CAS overlay. Since the response of different fractions (bands) to the assay is not known, the concentration should only be viewed as indicative. The resolution of the CAS overlay can be modulated with the application time. Although the spots develop generally shortly after applying the overlay and the reaction can be stopped, with longer application times weak spots may be visualized. This is an advantage over spraying techniques.

Siderophore diversity

Figure 3 shows patterns of partially purified pyoverdine from *P. aeruginosa* PA01 (lane 1) and PA6 (lane 2), partially purified pyoverdine from *P. fluorescens* ATCC 17400 (lane 3), purified pseudobactins from *P. putida* WCS 358 (lane 4), from *Pseudomonas* sp. BN7 (lane 5) and from *Pseudomonas* sp. B10 (lane 6), and purified deferrioxamine B (lane 7). The different patterns allow the identification of strains belonging to various groups, because of the diversity in siderophore migration in the IEF gel. In most cases the diversity spans the entire p*I* range used. This result shows that separation and detection can be achieved with siderophores of very different chemical nature, both peptidic and non-peptidic.

Comparison of crude supernatants and partially purified siderophores

When comparing CAS overlays of IEF gels run with crude supernatants and partially purified siderophores, some important differences were observed (results not shown). Crude supernatants were used for a rapid screening, but a partial purification eliminates salts, while concentrating the sample, which resulted in sharper bands. On the other hand, partial purification may also lead to a certain loss of classes of siderophores (e.g. the non-fluorescent acidic siderophore of *P. fluorescens* ATCC 17400 and its mutant 3G6), either because these siderophores are neither (or weakly) colored nor fluorescent and therefore insufficiently monitored in the elution step of this procedure or because these siderophores do not bind to the C_{18} column. In order to improve such a procedure, purification steps can be followed with the IEF–CAS method.

Effects of culture conditions on siderophore production

Effects of stress factors and culture conditions such as a low iron level or the presence of other metals on the regulation of siderophore production can easily be monitored with the IEF–CAS technique. Zinc was shown to cause an increase in pyoverdine production in *P. aeruginosa* 7NSK2 and in other fluorescent Pseudomonads (Höfte *et al.* 1993 and unpublished results).

Figure 4 illustrates that the signal for different *P. aeruginosa* siderophores does not respond similarly in the presence of various metal ions. Whereas the siderophore with neutral p*I* is only affected in cultures with Ni(II) ions (lanes 6 and 12), it can no longer be detected in cultures grown in the presence of Al(III) ions (lanes 7 and 13), which also induce a shift, apparently of the siderophore with alkaline p*I* to a higher p*I*. The acidic (non-fluorescent) *P. aeruginosa* siderophore, migrating up to the anode, which is most probably pyochelin, disappears when the bacteria were grown in the presence of Ni(II) or Al(III) (lanes 6 and 12, respectively, 7 and 13). For Ni(II) (as well as for other transition metal ions Co(II), Cu(II) and Mo(VI)) and pyochelin of *P. aeruginosa* PA01 this was also reported by Visca *et al.* (1992).

Conclusion

IEF with subsequent siderophore detection by CAS overlay provides one with information on the presence, diversity and regulation of production of these compounds. It allows straightforward screening of various isolates with detection of non-fluorescent siderophore bands, which would otherwise only be detected by biochemical analysis. The method is applicable both to pyoverdines (with a peptide moiety) and to certain non-peptidic siderophores. In view of the diversity of siderophore types, a first visualization of diversity contributes to directing subsequent biochemical analysis. Although it remains to be explained which structural differences account for the migration behavior, screening of siderophore diversity is greatly facilitated by the IEF–CAS technique.

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