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O-CAS, a fast and universal method for siderophore detection

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Abstract

In this work, the popular CAS assay for siderophore detection, based on the utilization of chrome azurol S, was redesigned and optimized to produce a new, fast, non-toxic, and easy method to determine a wide variety of microorganisms capable of siderophore production on a solid medium. Furthermore, this specific bioassay allows for the identification of more than one single siderophore-producing microorganism at the same time, using an *overlay* technique in which a modified CAS medium is cast upon culture agar plates (thus its name "O-CAS", for overlaid CAS). Detection was optimized through adjustments to the medium's composition and a quantifying strategy. Specificity of the bioassay was tested on microorganisms known for siderophore production. As a result, a total of 48 microorganisms were isolated from three different types of samples (fresh water, salt water, and alkaline soil), of which 36 were determined as siderophore producers. The compounds identified through this method belonged to both hydroxamate and catechol-types, previously reported to cause color change of the CAS medium from blue to orange and purple, respectively. Some isolated microorganisms, however, caused a color change that differed from previous descriptions.

Keywords: Chrome azurol S; Overlay; Siderophore; Universal method

1. Introduction

Microorganisms produce and secrete siderophores to sequester iron. These compounds can be defined typically as small molecules (often <1000 Da, although some siderophores are bigger) that are rapidly assembled through short, well-defined metabolic pathways. These molecules comprise lateral chains and functional groups that confer ligands a strong affinity (usually with $K_d > 10^{30}$ M^{-1}) to coordinate with the ferric ion (Neilands, 1981, 1995). Some siderophores (e.g., aerobactin) have lower and others (e.g., enterobactin) have higher affinities (Ratledge and Dover, 2000). Siderophores are typically produced by bacteria, fungi, and monocotyledonous plants in response to iron stress (Ratledge and Dover, 2000). Typically, microbial siderophores are classified as catecholates, hydroxamates, and α-carboxylates, depending on the chemical nature of their coordination sites with iron (Winkelmann, 1991, 2002). However, some siderophores like yersiniabactin are classified also as phenolates (Haag et al., 1993; Drechsel et al., 1995a), and others, in addition, as "mixed" (e.g., pyoverdins, produced by *Pseudomonas* species and containing both hydroxamate and catecholate functional groups) (Meyer and Hornsperger, 1978; Meyer and Stintzi, 1998).

Hydroxamates are produced by fungi and bacteria, whereas catecholates are produced exclusively by bacteria and comprise catechol and hydroxy groups as ligands. α-carboxylates are produced by the group of fungal zygomycetes (mucorales) and a few bacteria, such as *Rhizobium meliloti* and *Staphylococcus hyicus*, and coordinate iron through hydroxy and carboxyl groups (Drechsel et al., 1995b; Baakza et al., 2004).

In order to detect this kind of compounds, a variety of assays have been developed based on chemical properties [i.e., ferric perchlorate assays (Atkin et al., 1970), Csáky (Payne, 1994), Arnow (Arnow, 1937)], as well as on biological or functional properties [i.e., CAS universal assay (Schwyn and Neilands, 1987, modified by Ames-Gottfred et al., 1989, Milagres et al., 1999, and Machuca and Milagres, 2003)] and bioassays (Sung

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et al., 2001). Unlike chemical assays to detect siderophores, most of their functional counterparts are based on the CAS universal assay, in which growth medium includes not only the required nutrients but inhibitory compounds as well, thus making it unsuitable to cultivate some microorganisms such as fungi and Gram-positive bacteria. Variations to the CAS assay have been developed together with the CAS agar plate technique; however, these methods are laborious and inefficient as they can only detect one single microorganism type at the same time.

The objective of the present study was to develop an assay capable of surpassing previous methodological limitations, allowing for the detection of a variety of siderophore-producing microorganisms at once, while avoiding problems of toxicity and growth inhibition. The validity of the assay was tested using collection siderophore-producing microorganisms and the method was applied to detect wild producers from field samples of fresh water, salt water, and alkaline soil.

2. Materials and methods

2.1. Microorganisms and growth conditions

The fresh water sample was obtained at La Cruz Xalancocona site, in Mexico City, whereas salt water came from Tecolutla, Veracruz State, Mexico. The soil sample was taken at the former Lake Texcoco, outside Mexico City. All samples were stored in plastic containers at 4 °C prior to their use.

A variety of solid growth media was used to isolate microorganisms from each sample: Grimm–Allen medium (Grimm and Allen, 1954) for the ascomycetes group of fungi, modified M9 medium for fungi belonging to the zygomycetes, and nutrient agar for bacteria. The pH was adjusted for all growth media according to original values from field samples. For salt water isolates, previously filtered (0.45-μm pore size, 47-mm diameter, cellulose acetate, Whatman) seawater was used, in substitution of distilled water. Plates were maintained at 28 °C for 24 h. Microorganisms were preserved in a 20% (for bacteria) or 40% (for fungi) glycerol solution at −20 °C and characterized by preliminary inspection through microscopic observation and Gram-stain technique.

For siderophore production, isolates [fresh water (FW), salt water (SW), and alkaline soil (AS)] were cultivated in the media previously described, under iron deprivation, at 28 °C for 72 h. Sampling sites and types of samples (FW, SW, and AS) were chosen because ferric ions are found in low levels in these environments, thus making it simple to isolate microorganisms capable of producing compounds to sequester this metal. To achieve iron deprivation all media and glass materials were treated according to Cox (1994).

Collection microorganisms used as positive controls were *Aspergillus niger* ATCC 66876, *Rhizopus oligosporus* ATCC 22959, and *Bacillus cereus* ATCC 13061 strain, all are known for their siderophore production (Milagres et al., 1999, Baakza et al., 2004). These microorganisms belong to groups (fungi and Gram positive bacteria) presenting a problem of growth inhibition in the traditional CAS method (Schwyn and Neilands, 1987). Plates lacking microorganisms were used as negative controls at this point.

2.2. O-CAS assay

CAS medium was prepared according to Schwyn and Neilands (1987), although only as a means to reveal changes, without the presence of nutrients. The medium for a liter of *overlay* was as follows: Chrome azurol S (CAS) 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 mL. Agarose (0.9%, w/v) was used as gelling agent.

Siderophore detection was achieved after 10 mL (standard, 80 mm diameter Petri dishes) or 30 mL (long-size, 150 mm diameter Petri dishes) overlays of this medium were applied over those agar plates containing cultivated microorganisms to be tested for siderophore production. After a maximum period of 15 min, a change in color will be observed in the overlaid medium, exclusively surrounding producer microorganisms, from blue to purple (as described in the traditional CAS assay for siderophores of the catechol type) or from blue to orange (as reported for microorganisms that produce hydroxamates). Assay specificity was evaluated by repeating the above mentioned protocol with non-deferrated media (thus, lacking induction of siderophore production). All these experiments were made at least three times with three replicates for each one.

2.3. Chemical determination of produced siderophores

Chemical assays were performed to test the results obtained with the O-CAS method, as follows: for hydroxamate detection, the $FeCl_3$ assay (Neilands, 1981) was used; to detect catechols, the Arnow assay (Arnow, 1937); and the Shenker assay to detect carboxylates (Shenker et al., 1992). Quantification was made using a Lambda 35 Spectrometer (Perkin Elmer Instruments). All the assays were made at least three times with two replicates for each one.

2.4. Recovery of siderophore-producing microorganisms

Four kinds of microorganisms (non filamentous Gram-positive bacteria, Gram-negative bacteria, actinomycetes, and yeasts) were isolated and showed a positive response to the test, producing a variety of siderophores; these were recovered from the original culture medium by puncturing through the CAS overlay (O-CAS), using a sterile toothpick. Microorganisms were incubated for 24 h in the appropriate liquid medium and under optimal conditions, according to their origin. Growth was determined by turbidity and inoculation in Petri plates. Siderophore production confirmation was achieved after repeating the O-CAS assay with those recovered microorganisms.

3. Results

3.1. O-CAS detection method

To confirm that the overlay technique was functional, three siderophore-producing microorganisms were used: *A. niger* (catechol producer), which produced a color change from blue

to purple in the growth medium within 1 h (Fig. 1A), *B. cereus* (hydroxamate producer), with a change of color in the culture medium from greenish-blue to orange (Fig. 1B), and *R. oligosporus* (carboxylate producer) that changed the medium to a light yellow color (Fig. 1C) in 1 h. No changes in medium coloration were observed when the overlay was added to plates without microorganisms. No changes could be detected when non-deferrated media were used for microbial growth.

3.2. Isolation of microorganisms and siderophore production

Isolation of siderophore-producing microorganisms was made from field samples. The selection of microorganisms was based on the O-CAS assay, and the type of siderophore produced was assessed through chemical methods. The results obtained for some of the samples are shown in Fig. 2, and the overall results are presented in Tables 1, 2, and 3. Several microorganisms produced changes in medium coloration that

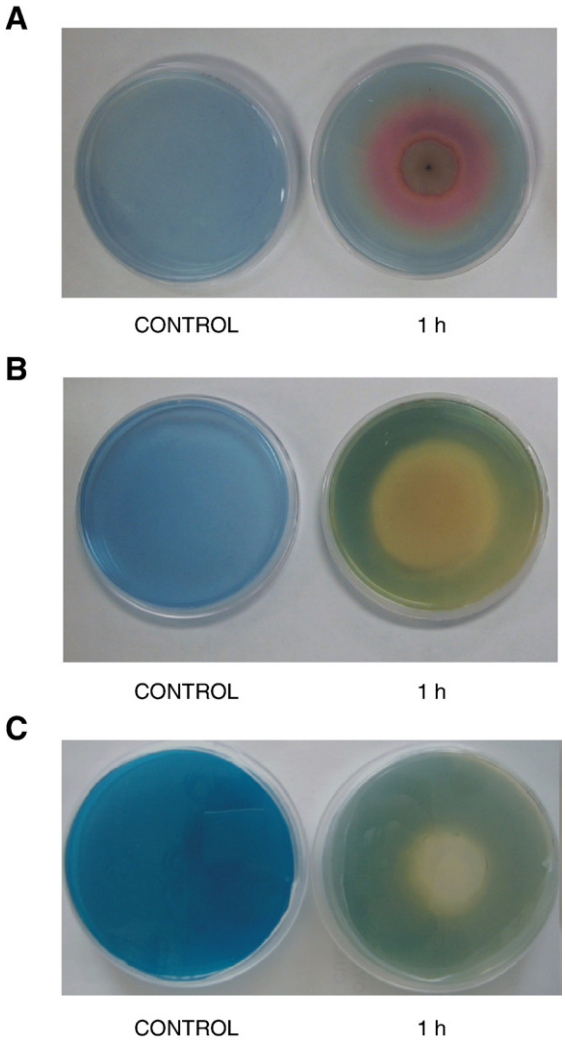

Fig. 1. O-CAS assay performed with collection strains. "Control" shows lack of reaction, and positive reaction is evaluated after 1 h. All the experiments were made at least three times with three replicates for each one. A: *Aspergillus niger*, catechol producer; B: *Bacillus cereus*, hydroxamate producer; C: *Rhizopus oligosporus*, carboxylate producer.

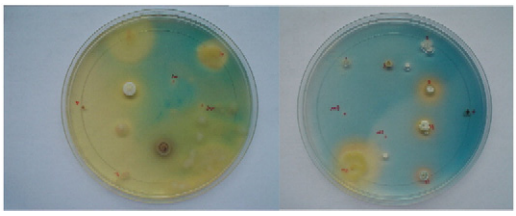

Fig. 2. O-CAS assay performed on two different samples. Different changes in the color of the medium can be appreciated.

are in agreement with previously reported studies. Hence, strain AS1 changed the color to purple (which corresponds to catechol-type siderophores, as mentioned above), whereas strains FW1, FW5, AS4, AS5, AS7, SW3, SW4, and SW10 rendered orange hues, which is consistent with chemical testing for hydroxamate-type siderophores. Other strains, such as FW8, SW9, SW13, and SW14, however, offered a reddish-orange coloration and, based on chemical testing, these organisms produced more than one type of siderophore (both hydroxamate and catechol). It is also possible that these organisms produced siderophores containing both catecholate and hydroxamate groups.

According to these tests, strains FW3, FW6, FW7, FW10, FW12, FW13, AS2, AS3, and SW6 also produced a variety of siderophore types (in this case hydroxamate and carboxylate), changing the color of the medium to a pale orange. Finally, some strains produced a yellow coloration, which has never been described before for CAS assays. In these cases, chemical tests showed that the corresponding siderophores belong to the carboxylate type. An interesting question derives from results for strains FW4, AS6, AS8, SW5, and SW7: these strains changed the medium to a yellow color (SW5 to orange), but surprisingly failed in all the chemical tests performed for siderophore detection. These cases will be discussed below.

Table 4 summarizes the results for isolated producer colonies from different samples (including both bacteria and fungi) and

Table 1
Comparison of the O-CAS method and chemical assays for fresh water samples

Colony	O-CAS [a]	$FeCl_3$ [b]		Arnow [b]	Shenker [b]
		420–450 nm	495 nm		
FW1	Orange	+	−	−	−
FW2	Yellow	−	−	−	−
FW3	Light orange	+	−	−	+
FW4	Yellow	−	−	−	−
FW5	Orange	+	−	−	−
FW6	Light orange	+	−	−	+
FW7	Light orange	+	−	−	+
FW8	Dark orange	+	+	+	+
FW9	Yellow	−	−	−	+
FW10	Light orange	+	−	−	+
FW11	Yellow	−	−	−	+
FW12	Light orange	+	−	−	+
FW13	Light orange	+	−	−	+
FW14	Dark yellow	+	−	−	+

All the assays were made at least three times with two replicates for each one.
[a] For the O-CAS assay, medium color change was considered.
[b] For chemical assays, "+" stands for siderophore production and "−" siderophore non-production.

Table 2
Comparison of the O-CAS method and chemical assays for alkaline soil samples

Colony	O-CAS [a]	$FeCl_3$ [b]		Arnow [b]	Shenker [b]
		420–450 nm	495 nm		
AS1	Purple	−	+	+	+
AS2	Light orange	+	−	−	+
AS3	Light orange	+	−	−	+
AS4	Orange	+	−	−	−
AS5	Orange	+	−	−	−
AS6	Yellow	−	−	−	−
AS7	Orange	+	−	−	−
AS8	Yellow	−	−	−	−

All the assays were made at least three times with two replicates for each one.
[a] For the O-CAS assay, medium color change was considered.
[b] For chemical assays, "+" stands for siderophore production and "–" siderophore non-production.

the type of siderophore they produced. Further characterization of the molecules produced by these strains is currently underway at our laboratories, as well as the molecular identification of producer microorganisms.

After the assay, all microorganisms randomly recovered from the plates were viable and able to reproduce the initial coloration change.

4. Discussion

The traditional methodology available for the detection of siderophore-producing microorganisms proposed by Schwyn and Neilands (1987) presents a problem of growth inhibition for Gram positive bacteria and fungi, due to the presence of hexadecyltrimetyl ammonium bromide (HDTMA) in the cultivation medium, which, at high concentrations, is toxic to these microorganisms. Ames-Gottfred et al. (1989) modified the traditional method to permit identification of various producer strains at the same time. This technique, however, still presents inhibition or poor growth of microorganisms, in addition to difficulties in the preparation of the medium. A later modification of the assay (Milagres et al., 1999), based on the use of Petri dishes in which the surface was split using half for the cultivation medium and half for the CAS detection medium, successfully avoided growth inhibition problems, while allowing development of all kinds of microorganisms. This method, however, is extremely laborious and results are slow to achieve; furthermore, it only permits identification of one single type of microorganism per plate.

Table 3
Comparison of the O-CAS method and chemical assays for salt water samples

Colony	O-CAS [a]	$FeCl_3$ [b]		Arnow [b]	Shenker [b]
		420–450 nm	495 nm		
SW1	Yellow	−	−	−	+
SW2	Yellow	−	−	−	+
SW3	Orange	+	−	−	−
SW4	Orange	+	−	−	−
SW5	Orange	−	−	−	−
SW6	Light orange	+	−	−	+
SW7	Yellow	−	−	−	−
SW8	Yellow	−	−	−	+
SW9	Dark orange	+	+	+	−
SW10	Orange	+	−	−	−
SW11	Orange	+	−	−	+
SW12	Orange	+	−	−	+
SW13	Dark orange	+	+	+	+
SW14	Dark orange	+	+	+	+

All the assays were made at least three times with two replicates for each one.
[a] For the O-CAS assay, medium color change was considered.
[b] For chemical assays, "+" stands for siderophore production and "–" siderophore non-production.

Table 4
Isolated microorganisms and type of siderophores obtained [a]

Microorganisms	Sample	Siderophores						
		A	B	C	D	E	F	G
Gram + bacteria	Fresh water	3	2	1	–	–	1	1
Gram − bacteria	Salt water, soil	4	2	–	5	1	–	–
Actinomycetes	Fresh water, alkaline soil	–	–	–	1	–	–	1
Fungi	Salt water	–	–		–	–	–	1
Yeast	Fresh water, salt water	1	1	–	5	–	–	–

[a] A: hydroxamate type siderophores, B: carboxylate type siderophores, C: catechol type siderophores, D: hydroxamate and carboxylate mix. E: hydroxamate and catechol mix, F: catechol and carboxylate mix, G: hydroxamate, catechol and carboxylate mix.

In the method hereby described, the CAS agar medium was used devoid of nutrients, only as an indicator of the presence of siderophores, and with 0.9% (w/v) agarose as gelling agent. The gel was spread as an overlay on the microorganisms (thus the name O-CAS for "overlaid CAS"). Growth of the microorganisms is performed with the most appropriate culture medium for each microbe (not necessarily over CAS medium, as the traditional method does). Moreover, the overlay is spread after the microorganisms have grown. Growth inhibition problems were surpassed in this way.

The lower concentration of gelling agent in the medium facilitates the diffusion of siderophores and shortens the time required for observation of results. The use of agarose (being a more refined product), as an agar substitute, also helps to control the process in a more efficient way. We have also successfully tried this method with 0.9% agar in the overlay (data not included); this would allow diminishing costs in the application of the method.

The specific chemical assays that were performed on selected samples showed a correspondence between coloration change in the medium and the functional group of each siderophore, thus verifying the validity of the method. Results from strains FW4, AS6, AS8, SW5, and SW7 could indicate a higher sensitivity to lower concentrations of siderophores, as used in this method, compared with the classical chemical tests or, alternatively, that we are in the presence of a new kind(s) of siderophore(s) not reported yet. Further characterization of the compounds produced by these strains will solve this question and discard the possibility of false positives occurrence using this method.

The O-CAS assay is a simple and universal method for the identification of more than one siderophore-producing strain at once, with results available in less than half an hour, and

suitable for any kind of microorganism and growth medium. It is specific for siderophore-producing microorganisms, as demonstrated by the absence of change in medium color for many strains during the selection process (some of them included in Fig. 2), microorganisms for which no siderophores were detected through the chemical methods used in this study. The method showed neither inhibition of growth, caused by reagents or the pH. Microorganisms can be easily recovered from their original medium after coloration has been produced, avoiding the need for replicas. Hence, this method could become a powerful first step for the isolation and selection of siderophore-producing microorganisms.

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