ISSN 0325-7541
INFORME BREVE Revista Argentina de Microbiología (2012) 44: 150-154

Siderophores of *Stenotrophomonas maltophilia*: detection and determination of their chemical nature

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ABSTRACT

Stenotrophomonas maltophilia is an emerging nosocomial pathogen. Despite the broad spectrum of syndromes associated with *S. maltophilia* infections, little is known about its virulence factors, including siderophore production. The aims of this work were to detect *S. maltophilia* siderophores and to determine their chemical nature. We studied 31 *S. maltophilia* isolates from device-associated infections, recovered over the period 2006-2011 at Hospital de Clínicas José de San Martin, Buenos Aires, Argentina, and the strain K279a, whose genome has been fully sequenced. The production of siderophores was screened by the chrome azurol S (CAS) agar assay, previously modified to detect siderophores in this species. When grown on modified CAS agar plates, all the clinical isolates and K279a were CAS-positive for siderophore production. In order to determine the chemical nature of siderophores, the Csáky (hydroxamate-type) and Arnow (catechol-type) assays were used. All *S. maltophilia* isolates produced catechol-type siderophores, but hydroxamate-type siderophores were not detected.
Key words: *Stenotrophomonas maltophilia*, siderophores, CAS agar, Csáky, Arnow

RESUMEN

Sideróforos de *Stenotrophomonas maltophilia*: detección y determinación de su naturaleza química. *Stenotrophomonas maltophilia* es un patógeno nosocomial emergente. A pesar de la variedad de infecciones que produce, poco se conoce acerca de sus factores de virulencia, incluida la producción de sideróforos. Nuestros objetivos fueron detectar sideróforos de *S. maltophilia* y determinar su naturaleza química. Se estudiaron 31 aislamientos provenientes de infecciones asociadas al uso de dispositivos médicos y la cepa K279a, cuyo genoma ha sido completamente secuenciado. Los aislamientos provenientes de infecciones se obtuvieron de pacientes asistidos en el Hospital de Clínicas José de San Martín (Buenos Aires, Argentina) en el período 2006-2011. Como método de tamizaje se empleó la técnica chrome azurol S (CAS) en placa, luego de implementar una modificación para detectar sideróforos en esta especie. Dicha modificación permitió detectar la producción de sideróforos en todos los aislamientos. La naturaleza química de los sideróforos se determinó mediante las técnicas de Csáky (hidroxamatos) y Arnow (catecoles). *S. maltophilia* produjo sideróforos de tipo catecol, mientras que no se detectaron sideróforos de tipo hidroxamato.
Palabras clave: *Stenotrophomonas maltophilia*, sideróforos, agar CAS, Csáky, Arnow

Stenotrophomonas maltophilia (formerly *Pseudomonas maltophilia* and *Xanthomonas maltophilia*) is a widespread environmental bacterium that has become a nosocomial pathogen of increasing importance. In fact, it is the third most common nosocomial non-fermenting bacteria. Infection occurs principally in immunocompromised subjects, and in patients exposed to invasive devices and/or broad spectrum antibiotics. *S. maltophilia* causes pneumonia associated with mechanical ventilation, catheter-related bacteremia, hemodialysis-related infections and urinary tract infections. Despite the broad spectrum of clinical syndromes associated with *S. maltophilia* infections, little is known about its virulence factors (3, 6).

Iron is an essential element required by microorganisms for many biological processes. Although Fe^{2+} is relatively soluble, in oxygen-rich environments iron is oxidized to Fe^{3+} which is extremely insoluble, making it difficult to acquire for microorganisms (11). Bacteria find iron limiting conditions in mammalian hosts, where free iron is limited and it is normally bound to sequestering proteins such as transferrin and lactoferrin. To counter iron starvation, bacteria have developed efficient iron uptake mechanisms that allow them to scavenge iron. One of the most common mechanisms is the synthesis and secretion of high-affinity ferric chelator compounds called siderophores (11). Siderophores

are low molecular weight compounds, and according to the functional groups they use as iron ligands they are classified into either hydroxamates or catecholes, while a few siderophores do not fall into these categories (2).

Siderophores are considered important virulence factors for many pathogens allowing the microorganism to survive in the host. Different reports have been published concerning the ability of *S. maltophilia* to synthesize siderophores. Using the universal CAS assay, Minkwitz and Berg (9) found that *S. maltophilia* isolates synthesized little amounts of siderophores, while Dunne *et al*. (8) reported that no detectable siderophores were produced by *S. maltophilia* W81. Chhibber *et al.* found ornibactin as *S. maltophilia* siderophore (5), while Ryan *et al.* mentioned that *S. maltophilia* K279a and R551-3 produce the catechol-type compound enterobactin based on their recently sequenced genomes (13). The aims of this work were to detect *S. maltophilia* siderophores and to determine their chemical nature.

We have studied *S. maltophilia* isolates recovered from patients with device-associated nosocomial infections, at Hospital de Clínicas José de San Martín, in Buenos Aires, Argentina, between January 2006-March 2011. Their sources are outlined in Table 1. We have also included strain *S. maltophilia* K279a isolated from the blood of an oncologic patient. K279a, whose genome is fully sequenced (http://www.sanger.ac.uk/Projects/S_maltophilia/), was a kind gift from Dr. Maxwell Dow (3). Isolates were identified as *S. maltophilia* by conventional bacteriological tests. Identification was confirmed by using API 20NE (bioMèrieux, Marcy l'Etoile, France). Isolates were kept frozen at -20 °C in 15 % glycerol. Before use, bacteria were cultured on tryptone soy agar (TSA, Oxoid Ltd, Basingstoke, Hampshire, UK) for 24 h at 35 °C.

The production of siderophores was screened initially by the universal chrome azurol S (CAS) agar assay described by Schwyn and Neilands (14) and modified by Armstrong *et al.* (1). The method is based on the fact that the dye chrome-azurol S incorporated into the agarized medium can form stable complexes with iron, resulting in a blue-green medium. When a chelator, such as a siderophore, removes the iron from the complex, the medium turns orange.

Modified CAS agar assay uses Stainer-Scholte (SS) minimal medium plus CAS-iron dye reagent (1, 14). We found that *S. maltophilia* isolates grew poorly in such condition, but its growth proficiency improved with the addition of 0.1 % (w/v) casamino acids (Difco laboratories, Detroit, MI, USA) (SSC). A total of 31 local isolates and the strain K279a were screened for the production of siderophores by growing them on plates containing agarized SSC plus CAS reagent (SSC-CAS). In these conditions only 4 clinical isolates were weakly positive, showing a small orange halo around the bacterial colony (≤ 4.0 mm) (Figure 1a, Table 1), while the positive control strain *Acinetobacter baumannii* ATCC19606 did produce a large halo (11.0 mm) (7). Since casamino acids is a source of iron, these negative results could reflect the existence of iron levels able to inhibit siderophore synthesis. Therefore, in order to achieve iron starvation and stimulate siderophore production, we used the specific Fe^{2+} chelator 2,2'-dipyridyl (Dip, Sigma Aldrich, Steinheim, Germany). Briefly, 60.5 mg of CAS (Sigma Aldrich, Steinheim, Germany) were dissolved in 50 ml of deionised water, and mixed with 10 ml of a Fe^{3+} solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). While stirring,

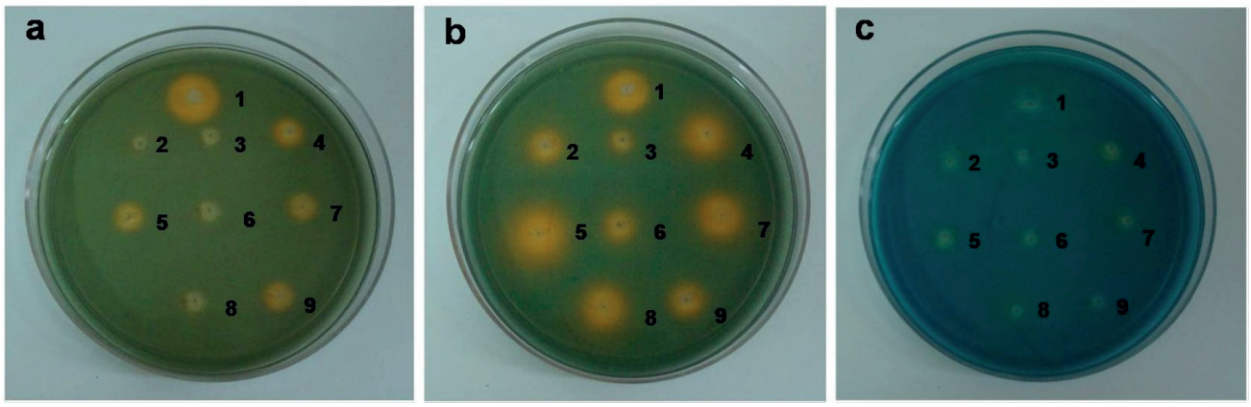

Figure 1. Production of siderophores screened by the CAS agar assay on plates containing a) SSC-CAS, b) SSC-CAS supplemented with 100 µM Dip, and c) SSC-CAS supplemented with 100 µM $FeCl_3$. 1: *Acinetobacter baumannii* ATCC19606; 2: Sm13; 3: Sm11; 4: Sm64; 5: Sm30; 6: Sm29; 7: Sm40; 8: K279a; 9: Sm9.

Table 1. Siderophore production by *S. maltophilia* clinical isolates

S. maltophilia Isolate	Isolate source [1]	CAS agar assay[7]		Arnow assay[8]	Csáky assay[9]
		SSC-CAS	SSC-CAS-DIP		
Sm9	Urine [2]	(+)	+	+	-
Sm10	Tracheal aspirate [6]	-	+	+	-
Sm11	Urine [2]	-	+	+	-
Sm13	Blood [3]	-	+	+	-
Sm14	Renal biopsy [4]	-	+	+	-
Sm15	Peritoneal fluid [5]	-	+	+	-
Sm17	Blood [3]	-	+	+	-
Sm18	Blood [4]	-	+	+	-
Sm19	BAL[6]	-	+	+	-
Sm20	BAL[6]	-	+	+	-
Sm26	BAL[6]	-	+	+	-
Sm27	BAL[6]	-	+	+	-
Sm28	BAL[6]	-	+	+	-
Sm29	Tracheal aspirate [6]	-	+	+	-
Sm30	Blood [3]	(+)	+	+	-
Sm31	Tracheal aspirate [6]	-	+	+	-
Sm32	Tracheal aspirate [6]	-	+	+	-
Sm33	Blood [3]	-	+	+	-
Sm34	Blood [3]	-	+	+	-
Sm35	Tracheal aspirate [6]	-	+	+	-
Sm36	BAL[6]	-	+	+	-
Sm37	Tracheal aspirate [6]	-	+	+	-
Sm38	BAL[6]	-	+	+	-
Sm39	BAL[6]	-	+	+	-
Sm40	BAL[6]	(+)	+	+	-
Sm 41	Tracheal aspirate [6]	-	+	+	-
Sm42	Tracheal aspirate [6]	-	+	+	-
Sm 61	Blood [3]	-	+	+	-
Sm 62	Tracheal aspirate [6]	-	+	+	-
Sm 63	Blood [3]	-	+	+	-
Sm 64	Tracheal aspirate [6]	(+)	+	+	-
K279a	Blood	-	+	+	-

[1]Sources of local *S. maltophilia* isolates from nosocomial infections associated to medical devices: [2]urinary catheter, [3]vascular catheter, [4]hemodialysis, [5]peritoneal dialysis and [6]mechanical ventilation (BAL: bronchoalveolar lavage). [7]For CAS agar assay: –, represents siderophore non-production; (+), ≤ 4.0 mm orange zone; +, 4.5 to 11.0 mm orange zone. For [8]Arnow and [9]Csáky assays: –, represents siderophore non-production; +, siderophore production. Results are from a representative experiment.

this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA, Sigma Aldrich, Steinheim, Germany) previously dissolved in 40 ml water. The resulting dark-blue solution was autoclaved, cooled to 50 °C and mixed with 900 ml of autoclaved SSC medium supplemented with 100 µM Dip (pH 7.6) (SSC-Dip) and 1.5 % agar (SSC-CAS-Dip). Each plate received 20 ml of blue-green agar. *S. maltophilia* isolates were cultured on trypticase soy agar (Oxoid Ltd, Basingstoke,

Hampshire, England) for 24 h at 35 °C. Isolated colonies were inoculated into trypticase soy broth (Oxoid) and incubated overnight on a gyratory water bath shaker (Model G75, New Brunswick Scientific Co. Edison N J, USA) at 200 r.p.m. Cultures were centrifuged for 5 min at 10,000 r.p.m., cell pellets were resuspended in normal saline (NaCl 0.9 % w/v, NS), centrifuged and washed twice. The OD_{546} of cell suspensions in NS was adjusted to 0.2 and 100 µl of standardized suspensions were used to inoculate SSC-Dip medium. After incubation for 48 h at 35 °C with shaking, cultures were centrifuged, the cell pellets were resuspended in NS and the OD_{546} was adjusted to 1.2. The standardized suspensions were stab inoculated onto CAS agar plates and incubated at 35 °C for 48 h. Siderophore production was recorded as the diameter of the orange halo produced by the colony.

When grown on SSC-CAS-Dip all the clinical isolates studied and the strain K279a produced an orange halo (4.5 to 11.0 mm) (Figure 1b). On the other hand, neither the *S. maltophilia* isolates nor *A. baumannii* ATCC19606 produced orange halos when 100 µM $FeCl_3$ was added to the SSC-CAS medium (Figure 1c).

In order to determine the chemical nature of siderophores produced by *S. maltophilia* the Csáky and Arnow assays were used (2). Briefly, *S. maltophilia* isolates were cultured in liquid SSC-Dip to achieve the iron limiting condition, or in SSC with 100 µM $FeCl_3$ (SSC-Fe) for iron-replete condition. Both media were inoculated with 100 µl of standardized suspensions as described above. Cultures were grown to stationary phase for 48 h at 35 °C with shaking. The OD_{546} of *S. maltophilia* isolates grown in SSC-Dip were similar (DO 0.700 ± 0.095) and lower than those obtained in SSC-Fe (DO 1.200 ± 0.120), showing that sufficient iron starvation to induce siderophore synthesis was achieved (2). Supernatants for siderophore assays were obtained by centrifugation at 10,000 r.p.m. for 5 min.

Bordetella bronchiseptica CCUG7865 which produces alcaligin siderophore (hydroxamate) was used as a positive control for the Csáky test (10, 12). *B. bronchiseptica* CCUG7865 gives a positive result when grown under the iron limiting condition; however, no hydroxamate compounds could be detected in any of the *S. maltophilia* supernatants tested in this study (Table 1). On the other hand, analysis of all the *S. maltophilia* culture supernatants obtained under iron starvation using the Arnow method showed positive results (Table 1), and the reaction was negative under full iron conditions, indicating the presence of catechol compounds. The same results were observed for the positive control strain *A. baumannii* ATCC19606 (7).

All assays were carried out at least in triplicate and repeated three times. Due to the fact that glass is an ion-exchange surface, long-term problems are caused by iron exchange from deeper layers. Therefore, concentrated nitric acid treatment for 4 h followed by extensive washing in deionised water was conducted to get rid of iron from labware in our experiments (2).

The universal chrome azurol S (CAS) agar assay has become a method widely used for screening siderophore production. This functional assay is exceptionally responsive and more convenient as screening method than assays based on chemical structures (Arnow and Csáky assays) because some siderophores do not fall into either hydroxamates or catecholes compounds. Using the CAS assay, Minkwitz and Berg (9) found that environmental and clinical *S. maltophilia* isolates synthesized little amounts of siderophores (3-5 mm orange halo). The detection of siderophores in this study was optimized through modifications of the medium´s composition. In SSC-CAS medium only a few isolates were weakly positive (≤ 4.0 mm orange halo), while in SSC-CAS-Dip all the isolates were CAS-positive for siderophore production (4.5 to 11.0 mm orange zone) (Figure 1a and 1b). The free iron concentration of the culture medium affects siderophore production (14). Chatterjee and Sonti also reported that *Xanthomonas oryzae* pv. *oryzae* did not produce siderophores in peptone-sucrose agar-CAS medium, but addition of dipyridyl led to siderophore production (4). Our results showed that the detection of siderophore production by *S. maltophilia* requires the addition to the CAS medium not only of casamino acids but also of dipyridyl (Table 1).

Since most siderophores are either hydroxamates or catechols, we performed the Csáky and Arnow assays to determine the chemical nature of siderophores produced by *S. maltophilia*. Analysis of iron-deficient culture supernatants using the Csáky method did not show hydroxamate compounds under these experimental conditions (Table 1). This result is not in agreement with that of Chhibber *et al.* who found ornibactin as *S. maltophilia* siderophore (5). These authors reported that the five clinical isolates cultured under iron-deplete conditions in M9 medium were positive for the production of ornibactin when screened by Gibson and Magrath's modified method (1969) and negative for the production of pyochelin by Arnow's method. However, these methods detect

hydroxamates and catechols compounds, respectively, and further characterization is needed for assignment of the specific siderophore. Furthermore, they did not mention positive controls and did not perform the assays in the presence of iron.

On the other hand, Ryan *et al.* mentioned that *S. maltophilia* K279a and R551-3 produce the catechol-type compound enterobactin based on their recently sequenced genomes (13). The genome of *S. maltophilia* K279a encodes the Smlt2817, Smlt2818 and Smlt2822 proteins, which are putative enterobactin synthasa component A, F and C, respectively, suggesting enterobactin production in this species (http://www.sanger.ac.uk/Projects/S_maltophilia/). Accordingly, the Arnow assay showed that all local *S. maltophilia* isolates and K279a produced catechol-type siderophores (Table 1).

Acknowledgements: the authors thank Maxwell Dow, Biomerit Research Centre, Department of Microbiology, Biosciences Institute, National University of Ireland, Cork, Ireland, for providing K279a strain. This research was supported by grant 20020100100 773 from UBACYT, Argentina.

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Recibido: 17/4/2012- Aceptado: 6/7/2012