ARTÍCULO ORIGINAL

ISSN 0325-7541
Revista Argentina de Microbiología (2009) 41: 73-78

Quorum sensing signal profile of *Acinetobacter* strains from nosocomial and environmental sources

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ABSTRACT

A set of 43 strains corresponding to 20 classified and unclassified genomic *Acinetobacter* species was analyzed for the production of typical N-acyl homoserine lactone quorum sensing molecules in culture broths. A large percentage of the strains (74%) displayed quorum sensing signals that could be separated into three statistically significantly different chromatographic groups ($p < 0.001$) based on their retention factor in TLC, i.e. Rf1 (0.22 ± 0.02); Rf2 (0.40 ± 0.02) and Rf3 (0.54 ± 0.02). Noteworthy, 63% of the strains tested produced more than one quorum signal. The frequency of signal appearance was Rf3 > Rf2 > Rf1. None of the three signals could be specifically assigned to a particular species in the genus; furthermore, no distinction could be made between the quorum sensing signals secreted by typical opportunistic strains of the *A. calcoaceticus-A. baumannii* complex, isolated from patients, with respect to the other species of the genus, except for the Rf1 signal which was present in all the QS positive strains belonging to this complex and DNA group 13 TU. In conclusion, quorum sensors in *Acinetobacter* are not homogenously distributed among species and one of them is present in most of the *A. calcoaceticus-baumannii* complex.

Keywords: *Acinetobacter*, quorum sensing, acyl homoserine lactone, nosocomial strains, environmental strains

RESUMEN

Perfil de sensores de quórum en cepas nosocomiales y ambientales de *Acinetobacter*. Se analizó la producción de moléculas típicas de N-acil homoserina lactona con actividad de *quorum sensing* en cultivos líquidos de un grupo de 43 cepas correspondientes a 20 especies genómicas clasificadas y no clasificadas de *Acinetobacter*. Un porcentaje alto de las cepas (74%) mostraron señales de *quorum sensing* que pudieron ser separadas en tres grupos cromatográficos significativamente diferentes entre sí ($p < 0,001$) sobre la base de sus factores de retención en TLC, a saber: Rf1 (0.22 ± 0.02); Rf2 (0.40 ± 0.02) y Rf3 (0.54 ± 0.02). Es de notar que 63% de las cepas ensayadas produjeron más de una señal de *quorum*. La frecuencia de aparición de las señales fue Rf3 > Rf2 > Rf1. Ninguna de las tres señales pudo ser asignada a una especie en particular dentro del género; es más, no se encontró diferencia entre las señales producidas por las cepas típicamente oportunistas (complejo *A. calcoaceticus-A. baumannii)* aisladas de pacientes respecto de las producidas por otras cepas del mismo género, excepto para el caso de Rf1, que se encontró presente en todos los aislamientos *quorum sensing* positivos del mencionado complejo y en las cepas del grupo de DNA 13TU. En conclusión, los sensores de quórum en *Acinetobacter* no están homogéneamente distribuidos entre especies y uno de ellos (Rf1) está presente en la mayoría de los miembros del complejo *calcoaceticus-baumannii.*

Palabras clave: *Acinetobacter*, sensores de quórum, acyl homoserina lactona, cepas nosocomiales, cepas ambientales

INTRODUCTION

Bacteria of the genus *Acinetobacter* can be isolated from environmental and clinical sources. Since the 1970s, strains belonging to this genus have emerged as multiresistant opportunistic nosocomial pathogens. Over the past two decades, the taxonomy of the genus *Acinetobacter* has undergone extensive revision (4, 10), and at least 32 named and unnamed species have been described. Within the genus *A. baumannii* and *Acinetobacter* genomic species 3 and 13 sensu Tjernberg and Ursing (13TU), which are clinically relevant, and the environmental species *A. calcoaceticus* are genetically closely related and phenotypically similar, which has led to the proposal to lump them together in the so called *A. calcoaceticus–A.baumannii* complex. *A. baumannii* is clinically the most important species, causing bacteremia, pneumonia, meningitis, urinary tract infections and surgical wound infections in hospitalized patients worldwide (5). It is important to notice that, since *Acinetobacter* genomic species 3 and 13TU are difficult to distinguish from *A. baumannii* by routine microbiological identification systems, the clinical role of these species may be underestimated. *A. baumannii* isolates are frequently resistant to a wide range

of antibiotics, and the frequency of multidrug-resistance among clinical isolates is rapidly increasing, making the available therapeutic options limited (14, 16). Recent advances have shown that a potential strategy to address the problem of multi-drug resistance is to target the global properties of infective organisms instead of harming each individual cell. Bacteria are indeed often involved in coordinated activities within a group, and many gram-negative bacteria produce N-acyl homoserine lactones (AHSLs) as signaling molecules in quorum sensing (QS) regulatory system (1, 7, 24). QS involving diffusible signals other than AHSLs are present in bacteria as well (1, 25), but they are not considered in this study.

It has been shown that virulence is among the functions regulated by QS, which therefore is a target of numerous efforts to circumvent its deleterious effects. Hence, interference with QS-based inter-cellular communication has become the basis of new therapeutic schemes (17, 18, 27).

We have previously detected QS signal molecules in *A. baylyi* ADP1 cultures, a typical soil-derived organism, and in culture supernatants of a few clinical *Acinetobacter* sp. isolates from Argentinean hospitals (8). It was therefore interesting to extend the investigation to a more representative and well-characterized sampling of the genus, with known origin and source.

The present study shows the distribution and diversity of homoserine-lactone based QS signals in a set of 43 *Acinetobacter* strains from hospitalized patients and environmental sources. The analysis was performed on culture supernatants, thereby assuring that only secreted molecules were analyzed.

Object: To describe QS signal distribution in a representative group of *Acinetobacter* species.

MATERIALS AND METHODS

Strains and growth conditions

Acinetobacter isolates were obtained from the culture collection of the Department of Infectious Diseases of Leiden University Medical Center (LUMC), Leiden, Netherlands (Table 1). A total of 43 strains, belonging to 19 classified named and unnamed species of *Acinetobacter* were investigated. The strains had been previously identified by DNA-DNA hybridization (2, 22) amplified ribosomal DNA restriction analysis (ARDRA; (6), and/ or amplified fragment length polymorphism (AFLP) analysis (9, 12). The latter two methods use restriction profiles, generated with five restriction enzymes or AFLP genomic fingerprints and comparison to profiles of reference strains, to come to final species identification.

A. tumefaciens reporter strain NT1, containing plasmid pZLR4 that carries *traR* and a *traG::lacZ* reporter fusion (3) was provided by Prof. Klaas Hellingwerf, University of Amsterdam, the Netherlands. This reporter detects a broad range of AHSLs : 3-oxo substituted with acyl chain length from 4 to 12 carbons; 3-unsubstituted AHSL (with the exception of C4) and 3- hydroxy AHSL (C6, C8 and C10) with good sensitivity on TLC (3, 20). The strain was grown in glucose M9 medium (19) containing 30 µg/ml gentamycin to mid log phase at 28 °C. Cells were centrifuged at 12,000 *g* for 15 minutes at 4 °C and the pellet was resuspended in 1/20 of its original volume in glucose M9 medium containing 20% glycerol. This suspension was stored in 1 ml cryovials at – 80 °C until further use.

The *Acinetobacter* strains were first grown overnight on blood agar. A colony of each was then transferred to a 250 ml Erlenmeyer flask containing 30 ml of a mineral medium of the following composition: 37 mM NH_4Cl, 0.81 mM Mg SO_4, 1.8 µM $FeSO_4$.7 H_2O, 68 µM $CaCl_2$, 11 mM KH_2PO_4, 95 mM, Na_2HPO_4 and 1 ml (per l) of a solution containing 50 g EDTA, 2.2 g $ZnSO_4$.7 H_2O, 1.6 g $CuSO_4.5H_2O$, 5 g $MnCl_2.4H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 50 mg H_3BO_3, 10 mg KI and 50 mg $CoCl_2.6H_2O$. Carbon source added to this medium was 1% lactic- plus 10 g/l acetic acid (MMA medium). Final pH of all media was 6.9. Flasks were incubated at 30 °C with agitation (250 rpm) for 12 hours.

Sample preparation

Cultures were centrifuged at 12,000 *g* for 10 min and the resulting supernatants were filtered through 0.22 µm PVDF membrane filters (Millipore). Supernatants were then extracted twice with 30 ml of ethyl acetate; subsequently the extracts were pooled, dried on anhydrous Na_2SO_4 and the solvent evaporated in a rotary evaporator at 40 °C. The extracts were then resuspended in 100 µl methanol and kept frozen at –20 °C. Fresh MMA medium (30 ml) was similarly extracted and used as a control. A solution of 2 mg/ml octanoyl-L-homoserine lactone (C8-HSL, Fluka) in methanol was used as a reference compound.

TLC separation of extracts

Ten µl of each of the resuspended extracts, 2 µl of C8-HSL reference solution and 10 µl of the control extracts were run on normal phase silicaGel GF 254 plates (20 cm x 20 cm, Merck, Darmstad, Germany), and developed with a mixture of chloroform: methanol (95:5, v/v). Plates were dried at room temperature in a laminar flow cabinet.

Autoinducer detection assay

One ml of the frozen stock suspension of the *Agrobacterium* reporter strain and 200 µl of 20 mg/ml X-gal solution in dimethyl formamide were added to 100 ml of M9 medium with 0.9% agar at 40 °C. The mixture was immediately poured over the dried TLC plates. After cooling, plates were placed in tightly sealed plastic boxes with a moist atmosphere and incubated at 28 °C for 24 h. Retention factors (Rf) were calculated for each identified signal, on the basis of the blue stain due to X-gal hydrolysis. The amount of C8-HSL required to produce an easily detectable spot with NT1 / pZLR4 reporter strain is in the order of 40 pmole (3, 20).

Statistical analysis was performed with Statistix 9.0 (http// www.statistix.com).

RESULTS

An overview of the QS signal results is given in Table 1 and a portion of the TLC profiles of the strains studied is shown in Figure 1. Using the *A. tumefaciens* NT1/pZLR4 reporter system, 32 strains (74%) were positive for QS signals, as evidenced by displaying at least one spot on TLC. Next, the Rf values of the QS signals identified in the 32 positive strains and their multiplicity were analyzed. All signaling molecules displayed an Rf value between 0.17 and 0.60, with a non-random distribution within this range. Moreover, it was possible to define three significantly different non-overlapping groups, Rf1 (0.22 ± 0.02), Rf2 (0.40 ± 0.02) and Rf3 (0.54 ± 0.02), as shown by one-way ANOVA ($p < 0.00$, Table 2) and Tukey test (MSD: 0.019).

Table 1. Description of *Acinetobacter* strains and QS signals

Species	Strain designation	Origin	Rf1	Rf2	Rf3
A. calcoaceticus	RUH 944[1]	I.V. catheter	0.24	-	-
	RUH 583[1]	Soil	0.22	-	0.56
	RUH 584[1]	Soil	0.23	-	-
A.baumannii	RUH 134[1]	Urine	0.23	0.43	0.56
	RUH 875[1]	Urine	0.21	-	0.55
	RUH 1063[1] (NCTC 7844)	Not known	-	-	-
Genomic species 3	RUH 509[1]	Bronchus	0.21	0.4	0.53
	RUH 56[1] (LMD 79.41)	Not known	-	-	-
	RUH 2204[1] (102[2])	Wound	0.25	-	-
	RUH 1163[1]	Toe web	-	-	-
A. haemolyticus	RUH 55[1] (LMD 70.9)	Not known	0.17	-	0.57
	RUH 415[1]	Pus	0.21	0.39	0.53
	RUH 2215[T, 1] (ATCC 17906[T])	Sputum	-	-	-
	RUH 44[1]	Air	0.26	-	0.6
A. junii	RUH 204	Blood	-	-	-
	RUH 2228[T] (ATCC 17908[T])	Urine	-	-	-
	RUH 2230 (178[2])	Water	-	-	0.54
A. johnsonii	RUH 2233[1] (92[b])	Urine	-	0.38	0.53
	RUH 2231[T, 1] (ATCC17909[T])	Gut	-	-	-
	LUH 540[1] (LMD 90.19)	Activated sludge	-	0.41	0.55
A. lwoffii	RUH 45[1]	Blood	-	0.4	-
	RUH 1104[1]	Skin	-	0.37	-
	RUH 303[1]	Door	-	0.4	-
Genomic species 10	RUH 2222[1] (198[1])	Urine	-	0.42	0.56
	RUH 2224[1] (ATCC 17924)	Not known	-	0.41	0.55
Genomic species 11	RUH 2234[1] (174[2])	Contact lenses	-	-	0.56
	RUH 2861[1] (CIP 63.46, ATCC 11171)	Not known	-	-	0.55
A. radioresistens	RUH 2226[1] (152[b])	Wound	-	0.4	-
	RUH 3517[1]	Feather pillow	-	0.4	0.54
Genomic species 13BJ/14TU	RUH 2216[1] (71[2])	Conjunctiva	-	0.41	-
Genomic species 13TU	RUH 503[1]	Urine	0.2	0.39	0.53
	RUH 2212[1] (165[2])	Urine	0.21	-	0.54
Genomic species 14BJ	LUH 1087	Conjunctiva	0.24	0.41	0.55
	LUH 1727[1] (513[3], CCUG 14816)	Wound	-	-	-
Genomic species 15TU	LUH 1090[1] (151[2])	Urine	-	-	-
Genomic species 16	LUH 1734[1] (1211[3])	Faeces	-	-	0.54
	LUH 1733[1](1011[3])	Skin	0.24	-	0.55
Genomic species 17	LUH 1735[1] (641[3], SEIP 2/87)	Wound	-	0.42	0.53
A. schindlerii	LUH 4764[4]	Pleura effusion	-	0.38	0.51
	LUH 5832[T,4]	Urine	-	-	-
A. ursingii	LUH 3792[T,4]	Blood	-	-	-
A. venetianus	LUH 3904[5]	Tar on the beach	-	0.39	0.51
A. beijerinckii	LUH 4759[T,6]	Wound	-	0.37	0.52

[1]Janssen *et al.* (9), [2]Tjernberg *et al.* (22), [3]Bouvet & Jeanjean (2), [4] Nemec *et al.* (12), [5]Vaneechoutte *et al.* (23), [6]Nemec *et al.* (13)

In addition, 12 strains displayed only one signal, either Rf1, Rf2 or Rf3 (37%), 6 strains displayed two: Rf1 and Rf3 (19%), 9 strains displayed Rf2 and Rf3 (28%) and 5 strains showed all three spots, Rf1, Rf2 and Rf3 (16%). It was also evident that Rf3 was the most frequently detected signal (24/32), followed by Rf2 (19/32) and Rf1(14/32).

Although not a single Rf could be assigned to a particular *Acinetobacter* species, Rf1 was predominantly found in *A. calcoaceticus-A. baumannii* complex and in

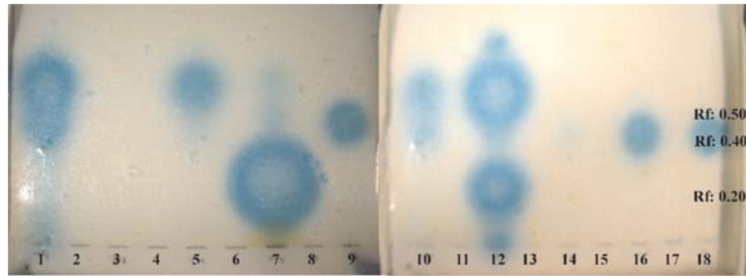

Figure 1. TLC separation of *Acinetobacter* culture extracts. Plates (silicaGel GF 254) were developed with a mixture of chloroform: methanol (95:5, v/v). After drying the plate, 1 ml of a suspension of the *Agrobacterium* reporter strain and 200 µl of 20 mg/ml X-gal solution in dimethyl formamide were added to 100 ml of M9 medium with 0.9% agar at 40 °C and poured on the TLC plate which was incubated at 28 °C for 24 h. Lane 1: LUH1087; 3: LUH1727; 5: LUH 1734; 7: RUH 509; 10: RUH 2222; 12: RUH 2212; 14: RUH 45; 16: RUH 2216. Lanes 2, 4, 6, 11, 13 and 15 left to allow space between samples; 8 and 17: control extract; 9 and 18: C8 HSL standard.

Table 2. Analysis of variance of Rf data from *Acinetobacter* isolations

Groups	*Size*	*Average*	*SD*	*Min*	*Max*	
Rf1	14	0.22	0.02	0.17	0.26	
Rf2	19	0.40	0.02	0.37	0.43	
Rf3	24	0.54	0.02	0.51	0.60	
Source of variations	*Square sum*	*Degrees of freedom*	*Medium square*	*F*	*P*	*Critical value for F*
Between groups	0.921	2	0.460991	1182.704	0.000000	5.021
Within groups	0.021	54	0.000389			
Total	0.943	56				

other incidentally infectious strains (*A. haemolyticus,* genospecies 14BJ and 16).

The intensity of the individual signals, measured as the diameter (in cm) of the X-gal derived stain did not follow an evident pattern (data not shown). However, one of the strains displayed an exceptionally intense Rf1 signal, compared with all others (i.e. strain RUH 509). In all cases, extracts prepared from uninoculated medium showed no signal/s.

DISCUSSION

A substantial number of the investigated *Acinetobacter* strains displayed QS signals belonging to three Rf groups**.** The presence of multiple QS signals in culture supernatants of gram-negative bacteria is not a rare finding. For instance in *Pseudomonas aeruginosa*, in which quorum sensing systems have been extensively characterized, two main AHSLs are produced (C4-HSL and 3oxo-C12-HSL), while the same signaling systems can synthesize other AHSLs in smaller amounts (3-oxo-C6-HSL and C6-HSL) as well (26). However, other *Pseudomonas* species have been found to display radically different profiles of QS signals. For instance, in *Pseudomonas aureofaciens,* the main QS signal was C6-HSL, which is also a regulator of the synthesis of the antibiotic phenazine, while in *Pseudomonas putida,* 3-oxo-C10-HSL and 3-oxo-C12-HSL are the main QS signals, and 3-oxo-C8-HSL and 3-oxo-C6-HSL are secreted in minor amounts (21).

It is interesting to note the rather limited diversity of the signals that are produced by the *Acinetobacter* isolates included in this investigation, which belong to 20 different genomic species and were collected from various sources. Moreover, Rf values showed little dispersion, as shown by the ANOVA analysis ($p < 0.001$). These results may indicate that AHSL synthases are randomly spread in the genus and are not limited to clinically relevant strains.

Since Rf1 appeared in all the (QS positive) clinically relevant strains analyzed and a few other strains (DNA group 13TU), this hydrophilic QS signal constitutes a candidate for a detailed structure study and interaction with the LuxR type receptor in the genus.

In a recent report (15), an AHSL from *A. baumannii* M2 was characterized as 3-HO-dodecanoyl-homoserine lactone (3 HO-C12-HSL). Although additional AHSLs were detected in culture supernatants of this strain, only one AHSL synthase gene was identified, suggesting that this synthase has low specificity and is capable of synthesize other QS signals. The Rf3 signal, identified in this study as the main *Acinetobacter* autoinducer, must be rather hydrophobic and could correspond to 3 HO-C12-HSL.

Preliminary genetic analysis of AHSL synthases performed on ten strains suggests that their DNA sequence may be quite variable. In fact, PCR amplification using full-length primers designed to amplify the sequence of the *A. baumannii* autoinducer synthase (*abaI,* GenBank accession number EU334497) or three different *luxI* internal consensus sequences, failed to amplify this gene from non- *baumannii* species that do produce QS signal(s) (R.H. González, unpublished results). As other synthases exist, besides the LuxI type, directing AHSL production (such as AinS of *Vibrio fischerii* and HdtS from *Pseudomonas fluorescens* (11)), their role in the genus *Acinetobacter* cannot be excluded. These observations open new questions about QS physiology in *Acinetobacter* to be answered with future research; the most relevant being the following:

-Is the *abaI* gene the only responsible one for the QS signals observed in the *Acinetobacter* species studied (or more general, in the genus?) and do the diversity of signals observed only respond to particular synthase specificity due e.g.: to substrate availability in the culture medium?

-Is the percentage of signal appeareance reflecting another mechanism involved in the QS signal excretion to the culture medium independently of the presence of AHSL synthase genes?

-Are there other AHSL´s synthase genes involved in the QS signal synthesis in the genus?

Acknowledgements: Professor Klaas J. Hellingwerf, from the Swammerdam Institute of Life Sciences, University of Amsterdam, Netherlands, provided reporter strains and counselling in experimental design; Dr. Ingela Tjernberg and Dr. P.J. Bouvet are acknowledged for their permission to use their strains. This work was supported by grant UBACYT B108 (University of Buenos Aires).

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Recibido: 30/09/08 – Aceptado: 31/03/09