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Identification of non-fermenting Gram-negative bacteria of clinical importance by an oligonucleotide array

Siou Cing Su,[1] Mario Vaneechoutte,[2] Lenie Dijkshoorn,[3] Yu Fang Wei,[1] Ya Lei Chen[4] and Tsung Chain Chang[1]

[1]Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

[2]Laboratory Bacteriology Research (LBR), Department of Clinical Chemistry, Microbiology and Immunology, Blok A, Ghent University Hospital, Ghent, Belgium

[3]Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

[4]Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung, Taiwan, ROC

Correspondence
Tsung Chain Chang
tsungcha@mail.ncku.edu.tw

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Many species of non-fermenting Gram-negative bacilli (non-fermenters) are important opportunistic and nosocomial pathogens. Identification of most species of non-fermenters by phenotypic characteristics can be difficult. In this study, an oligonucleotide array was developed to identify 38 species of clinically relevant non-fermenters. The method consisted of PCR-based amplification of 16S–23S rRNA gene intergenic spacer (ITS) regions using bacterial universal primers, followed by hybridization of the digoxigenin-labelled PCR products with oligonucleotide probes immobilized on a nylon membrane. A total of 398 strains, comprising 276 target strains (i.e. strains belonging to the 38 species to be identified) and 122 non-target strains (i.e. strains not included in the array), were analysed by the array. Four target strains (three reference strains and one clinical isolate) produced discrepant identification by array hybridization. Three of the four discordant strains were found to be correctly identified by the array, as confirmed by sequencing of the ITS and 16S rRNA genes, with the remaining one being an unidentified species. The sensitivity and specificity of the array for identification of non-fermenters were 100 and 96.7 %, respectively. In summary, the oligonucleotide array described here offers a very reliable method for identification of clinically relevant non-fermenters, with results being available within one working day.

INTRODUCTION

Non-fermenting Gram-negative bacilli (non-fermenters) are ubiquitous in the environment. Non-fermenters can cause a vast variety of infections (Dijkshoorn *et al.*, 2007; LiPuma *et al.*, 2007) and account for approximately 15 % of all Gram-negative bacilli cultured from clinical specimens (unpublished data of the National Cheng Kung University Hospital, Tainan, Taiwan). *Pseudomonas aeruginosa* is the most frequently isolated micro-organism, followed by *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* (Blondel-Hill *et al.*, 2007; LiPuma *et al.*, 2007; Schreckenberger *et al.*, 2007). Non-fermenters may differ in their pathogenic potential and transmissibility, and many are multidrug resistant (Schreckenberger *et al.*, 2007). For this reason, accurate identification of non-fermenters to species level is important for appropriate patient management.

In the diagnostic clinical microbiology laboratory, identification of non-fermenters relies mainly on phenotypic characteristics. A variety of commercial identification kits, such as API 20 NE (bioMérieux), VITEK 2 (bioMérieux) and Phoenix (Becton Dickinson), are being used for routine identification of these bacteria. Studies investigating the performance of these commercial identification systems have shown contradictory results (Funke & Funke-Kissling, 2004; Kiska *et al.*, 1996). In a recent study using API 20 NE, 54 % of non-*P. aeruginosa* non-fermenters were assigned to species level, 7 % to genus level and 39 % of isolates could not be discriminated at any taxonomic level, whilst with VITEK 2 the respective numbers were 53, 1 and 46 % (Bosshard *et al.*, 2006). Non-fermenters recovered

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS sequences generated in this study are listed in Table 2.

Details of the non-target strains tested are available with the online version of this paper.

from cystic fibrosis patients pose particular identification problems due to their phenotypic variation, atypical phenotypic characteristics and slow growth rates (Coenye *et al.*, 2005; Ferroni *et al.*, 2002).

Molecular identification techniques are emerging as alternatives for phenotypic identification methods. Among these, 16S rRNA gene sequencing is widely used (Kolbert & Persing, 1999; Patel, 2001; Vaneechoutte & De Baere, 2007). However, by 16S rRNA gene sequence analysis, only 92 % of non-*P. aeruginosa* non-fermenters were assigned to species level, with the remaining 8 % being assigned to genus level (Bosshard *et al.*, 2006). The intergenic spacer (ITS) region separating the 16S and 23S rRNA genes has been found to be a good candidate for bacterial identification (Chen *et al.*, 2004; Gürtler & Stanisich, 1996; Tung *et al.*, 2007). Recently, DNA array technology has been applied to identify a variety of micro-organisms with promising results (Fukushima *et al.*, 2003; Park *et al.*, 2005; Tung *et al.*, 2006). This study aimed to develop an oligonucleotide array based on ITS sequences to identify 38 species of non-fermenters with clinical relevance.

METHODS

Bacterial strains and DNA. A collection of 276 target strains (123 reference strains and 153 clinical isolates), representing 38 species of non-fermenters, were analysed (Table 1). Reference strains were obtained from: the American type Culture Collection (ATCC); the Belgian Co-ordinated Collections (BCCM/LMG); the Bioresources Collection and Research Center (BCRC); the Culture Collection, University of Göteborg (CCUG); the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ); and the Netherlands Culture Collection of Bacteria (NCCB). Clinical isolates were obtained from Ghent University Hospital (Belgium), Leiden University Medical Center (The Netherlands) and Kaohsiung Chang Gung Memorial Hospital (Taiwan). In order to assess the specificity of the array, a collection of 122 non-target strains belonging to 106 species other than the 38 target species were tested (see Supplementary Table S1, available in JMM online). All strains were cultured on sheep blood agar, incubated at 35 °C for 24–48 h and then used for DNA extraction by a boiling method (Millar *et al.*, 2000). DNA extracts of *Burkholderia pseudomallei* were obtained from clinical isolates recovered from patients with septicaemic melioidosis admitted to the Kaohsiung Veterans General Hospital, Taiwan (Chen *et al.*, 2006). These *Burkholderia pseudomallei* isolates were identified using biochemical test profiling (API 20 NE), by the presence of specific 16S RNA gene PCR amplicons (243 and 405 bp) and by the presence of a *fliC* gene PCR amplicon (267 bp) (Su *et al.*, 2007).

Design of oligonucleotide probes and array preparation. Forty-nine oligonucleotide probes (18–31mers) (Table 2), based on ITS sequences, were designed for identification of the 38 target species (Table 1). They included one positive control probe (designed from the 3′ end of the 16S rRNA gene) used to check for successful PCR and hybridization, three genus-specific probes used to identify *Acinetobacter* and *Pseudomonas*, one *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* complex-specific probe and 44 species-specific probes (Table 2). The *Pseudomonas*-specific probe (P3/P4) consisted of a mixture of probes P3 and P4 at an equimolar concentration. Probes used to identify the genera *Pseudomonas* and *Acinetobacter* and species in the *A. calcoaceticus*–*A. baumannii* complex were described in a recent study (Ko *et al.*, 2008). Between 5 and 15 additional bases of thymine were added to the 3′ ends of some probes to increase the hybridization signal (Brown & Anthony, 2000) (Table 2). A digoxigenin (DIG)-labelled irrelevant probe (5′-DIG-GCATATCAATAAGCGGAGGA-3) was spotted on the array and used as a position marker. The arrays (9 × 5 mm) were prepared with an automatic arrayer (model SR-A300; Ezspot) using a 400 μm-diameter solid pin as described previously (Tung *et al.*, 2006). The layout of different probes on the array is shown in Fig. 1.

Amplification of the ITS regions for array hybridization. The bacteria-specific universal primers 2F (5′-DIG-TTGTACACACCGCCCGTC-3′) and 10R (5′-DIG-TTCGCCTTTCCCTCACGGTA-3′) (Gürtler & Stanisich, 1996) were used to amplify the ITS regions, with each primer being labelled with a DIG molecule at its 5′ end. PCR was performed as described previously (Tung *et al.*, 2006).

Species determination by array hybridization. The procedures for pre-hybridization, hybridization (50 °C for 90 min) and colour development using anti-DIG antibodies have been described previously (Tung *et al.*, 2006). The hybridized spots (400 μm in diameter) could be read by the naked eye. A strain was identified as one of the species listed in Table 1 when both the positive control probe and the species-specific probe were hybridized (Table 2). A strain was identified as belonging to the genus *Acinetobacter* or *Pseudomonas* when the genus-specific probe, Aci2 or P3/P4, respectively (Table 2), was hybridized. In addition, a strain was identified as belonging to the *A. calcoaceticus*–*A. baumannii* complex when the complex-specific probe Acb2 was also hybridized (Ko *et al.*, 2008). A strain was identified as *Acinetobacter junii* only when both of probes Ajun2-1 and Ajun5 were hybridized. In contrast, a strain was identified as *Pseudomonas stutzeri* if at least one of the four probes Pstu1-3, Pstu2-2R, Pstu2-4 and Pstu3-7R designed for the species was hybridized, and as *Shewanella putrefaciens* if at least one of the two probes Sput1-3 and Sput3-4 was hybridized.

Discrepancy analysis. In cases where the array identification did not correspond with the original species name of the strain, the species identity of the strain was determined by sequencing of the ITS (Tung *et al.*, 2007) and the 16S rRNA gene. Amplification of the ITS by PCR was carried out as described above. The method of Relman (1993) was followed to amplify the 16S rRNA gene. PCR products were sequenced (Tung *et al.*, 2007) and the determined sequences were compared with reference sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). For ITS sequence comparison, final species identification was obtained only when the best-scoring ITS reference sequence had an identity of ⩾97 % with the query sequence (Chen *et al.*, 2004).

RESULTS AND DISCUSSION

Probe development

For most species, a single probe was sufficient to obtain identification, but multiple probes were needed to identify *A. junii*, *Acinetobacter lwoffii*, *P. stutzeri* and *Shewanella putrefaciens* (Table 2) due to intra- or interspecies sequence variation in the ITS regions. *A. junii* was identified only by simultaneous hybridization to two probes (Ajun2-1 and Ajun5), because *Acinetobacter johnsonii* LMG 1002 cross-hybridized with probe Ajun5 (Fig. 2, chip 8), whereas '*Acinetobacter venetianus*' CCUG 45561^T (a non-target species) cross-reacted with probe Ajun2-1 (Fig. 2, chip 46). Similarly, *Acinetobacter* gen. sp. 6 (Fig. 2, chip 6) and

Table 1. Non-fermenting Gram-negative bacilli used for sensitivity testing of the array

Species*	Reference strain	Clinical isolate†	Total no. of strains
Acinetobacter calcoaceticus	BCRC 11562, LMG 992, LMG 1046^T	LUH 9144, RUH 582, RUH 07658, RUH 08276, RUH 09548, RUH 4870	9
Acinetobacter baumannii	BCRC 10591^T, BCRC 15884, BCRC 15886, LMG 984	#010702, #050221, #090379, #350214	8
Acinetobacter gen. sp. 3	BCRC 15420, CCUG 26384, LMG 1035	LUH 8950, LUH 8951, LUH 8952, LUH 8953, #010621, #010631, #010632	10
Acinetobacter haemolyticus	BCRC 14852^T, BCRC 15887, LMG 997, LMG 1001	LUH 2520, LUH 2625, LUH 5708, LUH 6539, RUH 55, #030928	10
Acinetobacter junii	BCRC 14854^T	LUH 6981, #320514, #320515	4
Acinetobacter gen. sp. 6	BCRC 15421	LUH 286, LUH 4717, #Aci30, #Aci60	5
Acinetobacter johnsonii	BCRC 14853^T, BCRC 15888, LMG 1002	LUH 2386, LUH 7009, RUH 1526, RUH 2856, RUH 4941, #030934, #320521, #320527, #350473	12
Acinetobacter lwoffii	BCRC 14855^T, NCCB 83020	RUH 74, RUH 303, RUH 709, LUH 2626, LUH 749, #010604, #010605, #010606	10
Acinetobacter gen. sp. 9	LMG 985, LMG 1027, LMG 1300		3
Acinetobacter radioresistens	BCRC 15425^T, CCUG 26388, CCUG 34434	LUH 5679, LUH 3121, #080637, #080643, #080645	8
Acinetobacter gen. sp. 13TU	BCRC 15417	LUH 8948, LUH 8949, #Aci32, #Aci41, #080634	6
Acinetobacter gen. sp. 15TU	CCUG 26390	RUH 2930, LUH 8635, LUH 8943, #030943	5
Achromobacter xylosoxidans	BCRC 12839^T, BCRC 14180		2
Alcaligenes faecalis subsp. *faecalis*	ATCC 8748, ATCC 19018, ATCC 33585, ATCC 35655, BCRC 10828^T	#028, #043, #044, #045, #046	10
Burkholderia cepacia	BCRC 13208^T, BCRC 13906	#019, #065, #066	5
Burkholderia pseudomallei		ED01, FY03, 04, 05, VGH07, 16	6
Chryseobacterium indologenes	ATCC 29897^T, ATCC 49471	#029	3
Comamonas testosteroni	BCRC 10956, BCRC 14822^T	#076, #077, #091, #092, #093	7
Delftia acidovorans	ATCC 17438, ATCC 17455, ATCC 17476, BCRC 14819^T, BCRC 14830	#059, #096, #097, #098	9
Eikenella corrodens	BCRC 14415^T, CCUG 28283	#006, #007, #020, #049	6
Elizabethkingia meningoseptica	ATCC 13254, ATCC 13255, ATCC 33953, BCRC 10677^T	#012, #036, #037, #078	8
Moraxella atlantae	CCUG 10707, CCUG 31324, CCUG 46485	#010, #104/948	5
Moraxella bovis	BCRC 11229^T, CCUG 38236, LMG 1006	#024	4
Moraxella canis	CCUG 2153, LMG 11183, LMG 11190, LMG 11194^T	#027, #057, #089	7
Moraxella catarrhalis	BCRC 10628, BCRC 10629^T, BCRC 10630	#048, #085, #086, #087	7
Moraxella caviae	CCUG 355^T	#003	2
Moraxella nonliquefaciens	BCRC 11071, BCRC 11230^T, CCUG 37351, CCUG 47514	#023, #025, #058, #082	8
Moraxella osloensis	BCRC 10705^T, LMG 6916, LMG 6917, LMG 6918	#001, #004, #005, #008, #013, #050	10
Pseudomonas aeruginosa	ATCC 27853, BCRC 10944^T	B248/99, 04579, 06071, 06129, 07194-1, 07194-2, 07344, 07346, 7036, 9542	12

Table 1. cont.

Species*	Reference strain	Clinical isolate†	Total no. of strains
Pseudomonas alcaligenes	ATCC 53877, ATCC 55044, BCRC 13909^T, LMG 1224, LMG 6353	#063	6
Pseudomonas fluorescens	BCRC 10304, BCRC 10907, BCRC 11028^T, BCRC 13902, BCRC 13904, BCRC 13910, BCRC 16016	#026, #061, #067, #068	11
Pseudomonas mendocina	ATCC 25412, BCRC 10458^T	#031	3
Pseudomonas pseudoalcaligenes	ATCC 17443, BCRC 11902^T	#032, #033, #034, #064, #070, #094, #095	9
Pseudomonas putida	BCRC 10459^T, BCRC 10460, BCRC 10461, BCRC 14349, BCRC 14365	#038, #039, #060	8
Pseudomonas stutzeri	ATCC 17588^T, BCRC 14821, BCRC 15836, DSMZ 6082, DSMZ 6084, DSM 10701, DSMZ 50227, DSMZ 50238	#015	9
Ralstonia pickettii	BCRC 14093, BCRC 14820^T	#017, #052, #053, #054, #055, #056	8
Shewanella putrefaciens	ATCC 49138, ATCC 51753, BCRC 10596^T, BCRC 15549	#062, #073	6
Sphingomonas paucimobilis	BCRC 11672, BCRC 13893^T, BCRC 13954, BCRC 13955, LMG 2239, LMG 11151	#069, #079, #084	9
Stenotrophomonas maltophilia	BCRC 10737^T, BCRC 11901, BCRC 12495, BCRC 14110, BCRC 15550	#075	6
Total	123	153	276

*gen. sp., Genomic species.

†Clinical isolates with a prefix of '#' were identified by tDNA spacer fingerprinting (Baele *et al.*, 2000; Vaneechoutte *et al.*, 1998), whilst those with a prefix of 'LUH' or 'RUH' were identified by amplified rDNA restriction analysis (Dijkshoorn *et al.*, 1998) or whole-genome fingerprinting using amplified fragment length polymorphism analysis (Nemec *et al.*, 2001). Other clinical isolates were identified by API 20 NE (bioMérieux).

Table 2. Oligonucleotide probes used for identification of 38 species of non-fermenting Gram-negative bacilli

Micro-organism	Probe information					
	Code*	Sequence (5′→3′)†	Length (nt)	T_m (°C)	Location‡	GenBank accession no.
Positive control	PC§	TGGGGTGAAGTCGTAACAAGGTAGCCGTAttttttttt	29	65.8	1463–1491	AY616658
Acinetobacter spp.	Aci2‖	GAATCGAGCGTTTTGGTATATGAATTtttttttt	26	56.0	486–511	AY601823
A. calcoaceticus–*A. baumannii* complex	Acb2‖	GACTGGTTGAAGTTATAGATAAAAGATtttttttt	27	48.6	144–170	AY601823
A. calcoaceticus	Acal2‖	CATTGATCATGTCTTATTACTCCTTGTAGGttttt	30	56.2	584–613	AY601820
A. baumannii	Abau2‖	CGGTAATTAGTGTGATCTGACGAttttttttt	23	51.3	351–373	AY601823
Acinetobacter gen. sp. 3	Aun3-3‖	GATGAAGAATCGCACGGACAACAttttttttttt	23	58.6	560–582	AY601829
A. haemolyticus	Ahae3	GGCAACAAACTGGATGAGAGCGttttttttt	22	58.4	518–539	AY601831
A. junii	Ajun2-1	GCTGCTAGATAAAAAGATATGGCGttttttttttttt	24	53.7	165–188	AY601832
	Ajun5	AGGCACACATAGAACGAAGCTTGGAGtttttttt	26	60.8	346–371	AY601832
Acinetobacter gen. sp. 6	Aun6-5	GTGTTGCAGTTAGATGAAAGATTAATGCttttttttt	28	56.0	471–498	AY601833
A. johnsonii	Ajoh1-1	ATTTATTACTGATTGATGAAGGTTGAAGttttttt	28	53.0	520–547	AY601834
A. lwoffii	Alwo3	GTTTAAGGCTCAGAACTAAATTGACATTGA	30	57.8	419–448	AY601835
	Aun9-1	TAAGCGATCAAGTGATGAGATCCTAGAG	28	57.6	355–382	AY601836
A. radioresistens	Arad2	GCAACCTGTTGACTAACGTAGATTGAACttttttttt	28	57.7	464–491	AY601839
Acinetobacter gen. sp. 13TU	A13TU	GTGGGTAACGTCGACTATttttttttttt	18	41.3	586–603	AY601830
Acinetobacter gen. sp. 15TU	A15TU	TCGCAAGATTGATTGTTGAAGTGATT	26	57.9	493–518	AY601841
Achromobacter xylosoxidans	Axy2	GACCTGTGCCTGGTGGGATGTAGAttttttttt	24	60.9	315–338	EU014586
Alcaligenes faecalis subsp. *faecalis*	Afae5	CAACAGAACAAATTATGTTCAACGAAAGTTttttttttt	30	58.3	500–529	EU014606
Burkholderia cepacia	Bcep3R	CGGCAATGAGAAAAACTCGCACttttttttttt	22	58.4	139–160	EU014582
Burkholderia pseudomallei	Bmalpse1	ATGGATAATGTTTGATTGGGTTCGAGAtttttttt	27	59.7	341–367	EU014548
Chryseobacterium indologenes	Cind5R	TACTAATTTCTAGTGGGTTTTGTAttttttttt	24	45.2	118–141	EU014570
Comamonas testosteroni	Ctes3	CGAAAGATGCAGCCAAAGATATTCACttttttt	26	59.2	583–608	EU014531
Delftia acidovorans	Daci1	AGCGTGCTATAATATTTGACTCAACACTAAttttttt	30	56.6	331–360	EU014532
Eikenella corrodens	Ecor1	AGCATCCACACCTATCGGTAATCAGAttttttttt	26	58.9	57–82	EU014522
Elizabethkingia meningoseptica	Emen2	ACTGATACAAGTATACGAATAGAGCCAAAAttttttttt	30	55.9	442–471	EU014552
Moraxella atlantae	Matl2-2	ATAGCGATATAGAAAGTTAGCACTGTAGCCtttttttt	30	56.8	428–457	EU014598
Moraxella bovis	Mbov1	AATTTAAGTAAGATGAGTGGTCGGATAAAttttttt	29	55.7	425–453	EU014575
Moraxella canis	Mcan1-1	CCACCACTAAACATCAAAGCATCCAttttttttt	25	58.9	262–286	EU014611
Moraxella catarrhalis	Mcat5	GGCTCCACCAAGCAAGTTTAAACATCttttttttt	26	60.1	210–235	EU014604
Moraxella caviae	Mcav2	CCAACTAAACAATCAACCACTAAAGTAAAGAtttttttt	31	57.2	399–429	EU014537
Moraxella nonliquefaciens	Mnon2	AAAACTTTTAGTATTGATGATGATCGGAttttttttt	28	55.1	470–497	EU014585
Moraxella osloensis	Mosl3	CTCCACCATAATTAGCTTAACGTTATAAAGttttttttt	30	55.4	452–481	EU014577
Pseudomonas spp.	P3	GTTCTTTAAAAATTTGGGTATGTGATAGAA	30	55.4	321–350	EU014530
	P4	CAGTGACCAGATTGCTTGGGGTTATATtttttttt	27	59.5	445–471	EU014530
Pseudomonas aeruginosa	Paer2R	GGATCACGCAGCACACCAACAACAATtttttttt	26	64.6	139–164	EU014530
Pseudomonas alcaligenes	Palc2	TGCACGTTACGAATCTATAACCAGGTTtttttttt	27	58.3	459–485	EU014521

Table 2. cont.

Micro-organism	Probe information					
	Code*	**Sequence (5′→3′)†**	**Length (nt)**	**T_m (°C)**	**Location‡**	**GenBank accession no.**
Pseudomonas fluorescens	Pflu2	GGAAGCAGCCCGAAATTGGGTtttttttttttt	21	60.9	66–86	EU014587
Pseudomonas mendocina	Pmen1	GCGCTTCAGGGTAAGCCTGGTtttttttttttt	21	59.3	301–321	EU014574
Pseudomonas pseudoalcaligenes	Ppse4	TTCAGGTTTGTCCTGTTGAGTGCtttttttttttt	23	55.7	306–328	EU014553
Pseudomonas putida	Pput1	TGGGAGTTCTGCTCGAAAGATCAtttttttttt	23	57.6	425–447	EU014557
Pseudomonas stutzeri	Pstu1-3	AGAAGTAGACCGATGTGTTGCTTGCtttttttttt	25	57.7	373–397	AJ251910
	Pstu2-2R	CCACATCTACAGATCGGCCAAATtttttttttttt	23	56.8	180–202	CP000304
	Pstu2-4	TCCTGAGGTATGGAGAGTATTGATTGCttttttt	27	57.9	314–340	AJ251901
	Pstu3-7R	GTCTTGAAGCCTGCGCCACATAACttttttttttt	24	60.8	144–167	AJ251909
Ralstonia pickettii	Rpic2	TGGAAGATGTTCTCTGCCGTGACTtttttttttt	24	58.8	150–173	EU014524
Shewanella putrefaciens	Sput1-3	GAATGAAAAGCACAATAACGACAACATGAttttttt	29	60.3	417–445	EU014591
	Sput3-4	TAGAGCGAAACATCCCTCTAGCAGGTtttttttttt	26	59.7	69–94	EU014565
Sphingomonas paucimobilis	Spau2	TGAAGGTGTCGGCTTTGAAAGTGA	24	59.6	555–578	EU014520
Stenotrophomonas maltophilia	Smal3	ACATTCGATCTTTATACGCATCAGCACtttttttttt	27	58.7	338–364	EU014579

*Oligonucleotide probes were arranged on the array as indicated in Fig. 1.

†Multiples bases of thymine, indicated by 't', were added to the 3′ end of the probe. The underlined nucleotide indicates a single mismatch base that was intentionally incorporated into the probe to avoid cross-hybridization.

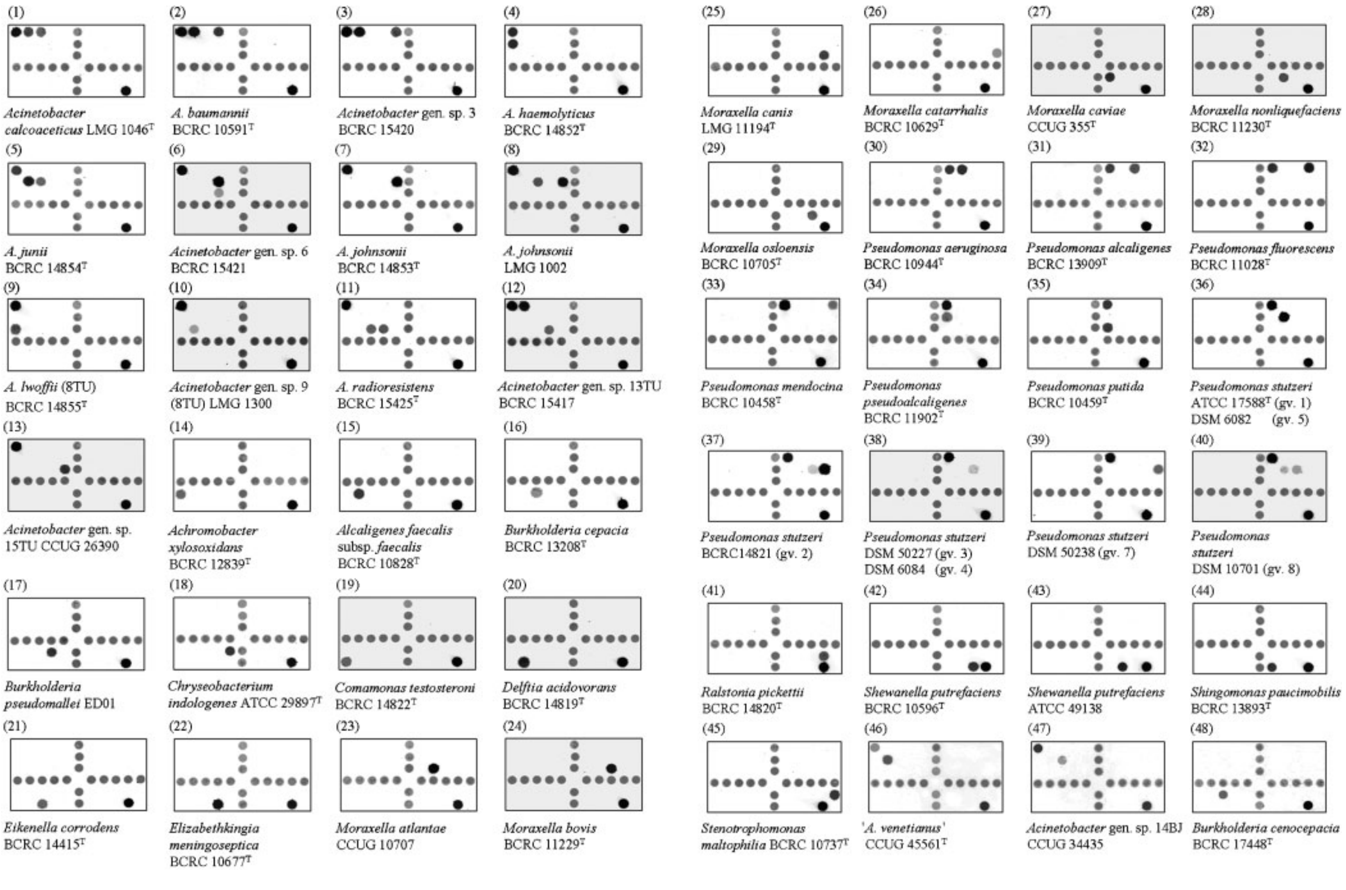
‡The location of probes is indicated by the nucleotide number of the ITS region except for the positive control probe.

§The positive control probe was designed from a conserved region in the 16S rRNA gene.

||Probes designed in a previous study (Ko *et al.*, 2008).

	1	2	3	4	5	6	7	8	9	10	11
A	Aci2	Acb2	Acal2	Abau2	Aun3-3	M	P3/P4	Paer2R	Palc2	Pflu2	Pmen1
B	Ahae3	Ajun2-1	Ajun5	Aun6-5	Ajoh1-1	M	Ppse4	Pstu1-3	Pstu2-2R	Pstu2-4	Pstu3-7R
C	Alwo3	Aun9-1	Arad2	A13TU	A15TU	M	Pput1	Matl2-2	Mbov1	Mcan1-1	Mcat5
D	M	M	M	M	M	NC	M	M	M	M	M
E	Axy2	Afae5	Bcep3R	Bmalpse1	Cin5R	M	Mcav2	Mnon2	Mosl3	Rpic2	Smal3
F	Ctes3	Daci1	Ecor1	Emen2	NC	M	Spau2	Sput1-3	Sput3-4	PC	NC

Fig. 1. Layout of oligonucleotide probes on the array (9×5 mm). Probe PC (F10) is the positive control probe designed from a conserved region in the 16S rRNA gene. Probe NC (D6) is the negative control (tracking dye only). Probe M is a DIG-labelled oligonucleotide that was used as a position marker. Genus-specific and *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex-specific probes are underlined. The sequences of the oligonucleotide probes are listed in Table 2.



(1) *Acinetobacter calcoaceticus* LMG 1046^T

(2) *A. baumannii* BCRC 10591^T

(3) *Acinetobacter* gen. sp. 3 BCRC 15420

(4) *A. haemolyticus* BCRC 14852^T

(5) *A. junii* BCRC 14854^T

(6) *Acinetobacter* gen. sp. 6 BCRC 15421

(7) *A. johnsonii* BCRC 14853^T

(8) *A. johnsonii* LMG 1002

(9) *A. lwoffii* (8TU) BCRC 14855^T

(10) *Acinetobacter* gen. sp. 9 (8TU) LMG 1300

(11) *A. radioresistens* BCRC 15425^T

(12) *Acinetobacter* gen. sp. 13TU BCRC 15417

(13) *Acinetobacter* gen. sp. 15TU CCUG 26390

(14) *Achromobacter xylosoxidans* BCRC 12839^T

(15) *Alcaligenes faecalis* subsp. *faecalis* BCRC 10828^T

(16) *Burkholderia cepacia* BCRC 13208^T

(17) *Burkholderia pseudomallei* ED01

(18) *Chryseobacterium indologenes* ATCC 29897^T

(19) *Comamonas testosteroni* BCRC 14822^T

(20) *Delftia acidovorans* BCRC 14819^T

(21) *Eikenella corrodens* BCRC 14415^T

(22) *Elizabethkingia meningoseptica* BCRC 10677^T

(23) *Moraxella atlantae* CCUG 10707

(24) *Moraxella bovis* BCRC 11229^T

(25) *Moraxella canis* LMG 11194^T

(26) *Moraxella catarrhalis* BCRC 10629^T

(27) *Moraxella caviae* CCUG 355^T

(28) *Moraxella nonliquefaciens* BCRC 11230^T

(29) *Moraxella osloensis* BCRC 10705^T

(30) *Pseudomonas aeruginosa* BCRC 10944^T

(31) *Pseudomonas alcaligenes* BCRC 13909^T

(32) *Pseudomonas fluorescens* BCRC 11028^T

(33) *Pseudomonas mendocina* BCRC 10458^T

(34) *Pseudomonas pseudoalcaligenes* BCRC 11902^T

(35) *Pseudomonas putida* BCRC 10459^T

(36) *Pseudomonas stutzeri* ATCC 17588^T (gv. 1)
DSM 6082 (gv. 5)

(37) *Pseudomonas stutzeri* BCRC14821 (gv. 2)

(38) *Pseudomonas stutzeri* DSM 50227 (gv. 3)
DSM 6084 (gv. 4)

(39) *Pseudomonas stutzeri* DSM 50238 (gv. 7)

(40) *Pseudomonas stutzeri* DSM 10701 (gv. 8)

(41) *Ralstonia pickettii* BCRC 14820^T

(42) *Shewanella putrefaciens* BCRC 10596^T

(43) *Shewanella putrefaciens* ATCC 49138

(44) *Shingomonas paucimobilis* BCRC 13893^T

(45) *Stenotrophomonas maltophilia* BCRC 10737^T

(46) '*A. venetianus*' CCUG 45561^T

(47) *Acinetobacter* gen. sp. 14BJ CCUG 34435

(48) *Burkholderia cenocepacia* BCRC 17448^T

Fig. 2. Hybridization results for 38 species of non-fermenters. Chip numbers are shown in parentheses and the corresponding probes hybridized on the arrays are indicated in Fig. 1. The cross-hybridization patterns of '*Acinetobacter venetianus*' CCUG 45561^T, *Acinetobacter* gen. sp. 14BJ CCUG 34435 and *Burkholderia cenocepacia* BCRC 17448^T are also shown (chips 46–48).

Table 3. Analysis of discrepant strains by sequencing of the ITS and 16S rRNA genes

Strain	Received as:	Genomic species or species identified by:			Best match
		Array hybridization	ITS sequence (% identity)*	16S rRNA gene sequence (% identity)	
LMG 1027	*Acinetobacter* genomic species 9	*Acinetobacter baumannii*	*Acinetobacter baumannii* (100) *Acinetobacter* gen. sp. 9 (68.7)	*Acinetobacter baumannii* (100) *Acinetobacter* gen. sp. 9 (96.4)	*Acinetobacter baumannii*
BCRC 10907	*Pseudomonas fluorescens*	*Pseudomonas putida*	*Pseudomonas putida* (NA) *Pseudomonas fluorescens* (83.9)	*Pseudomonas putida* (99.9) *Pseudomonas fluorescens* (97.3)	*Pseudomonas putida*
BCRC 14365	*Pseudomonas putida*	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* (NA) *Pseudomonas putida* (85.6)	*Pseudomonas fluorescens* (99.0) *Pseudomonas putida* (96.6)	*Pseudomonas fluorescens*
#003†	*Moraxella caviae*	(not identified)	*Moraxella caviae* (86.3)	*Moraxella cuniculi* (97.6)	*Moraxella* sp.

*NA, No corresponding ITS sequence of the species present in GenBank.
†A clinical isolate.

Acinetobacter radioresistens (Fig. 2, chip 11), in addition to their specific probes, cross-hybridized with probe A13TU used to identify *Acinetobacter* gen. sp. 13TU (a member of the *A. calcoaceticus*–*A. baumannii* complex), but neither one was misidentified as *Acinetobacter* gen. sp. 13TU as the complex-specific probe Acb2 was not hybridized.

Different genomovars of *P. stutzeri* (Guasp *et al.*, 2000) produced different hybridization patterns with the four probes Pstu1-3, Pstu2-2R, Pstu2-4 and Pstu3-7R designed for this species. For example, *P. stutzeri* ATCC 17588^{T} (genomovar 1) (Fig. 2, chip 36) and DSM 6082 (genomovar 5), in addition to the *Pseudomonas*-specific probe P3/P4, hybridized to probe Pstu1-3, whilst strain BCRC 14821 (genomovar 2) hybridized to another two probes (Pstu2-2R and Pstu2-4) (Fig. 2, chip 37). *A. lwoffii* and *Acinetobacter* gen. sp. 9 are synonyms (Tjernberg & Ursing, 1989), and identification of the organism was made if at least one of the two probes (Alwo3 and Aun9-1) was hybridized (Fig. 2, chips 9 and 10).

Identification of reference strains by the array

The hybridization patterns of the 38 species of non-fermenters on the arrays are shown alphabetically in Fig. 2. Of 123 reference strains belonging to the 38 target species, 120 hybridized to their respective oligonucleotide probes and were correctly identified. *Acinetobacter* gen. sp. 9 LMG 1027, *Pseudomonas fluorescens* BCRC 10907 (=ATCC 13430) and *Pseudomonas putida* BCRC 14365 (=ATCC 31800) were identified as *A. baumannii*, *P. putida* and *P. fluorescens*, respectively (Table 3). Discrepancy analysis by sequencing of the 16S rRNA genes revealed that all three identifications obtained by the array were correct. Identification of *Acinetobacter* gen. sp. 9 LMG 1027 as *A. baumannii* by the array was further confirmed by ITS sequencing. However, the identifications of *P. fluorescens* BCRC 10907 as *P. putida* and *P. putida* BCRC 14365 as *P. fluorescens* by the array could not be confirmed by ITS sequence comparison (sequence identities <97 %) (Table 3), due to the lack of corresponding ITS sequences in public databases. *P. putida* and *P. fluorescens* are phenotypically similar; several tests (gelatin, trehalose, inositol and lecithinase) could be used to differentiate the two micro-organisms, with *P. fluorescens* being positive for the four tests and *P. putida* being negative (Blondel-Hill *et al.*, 2007; Palleroni, 1984). In brief, all 123 reference strains, including the three strains that had been named incorrectly, were correctly identified by the array.

Identification of clinical isolates by the array

In this study, only clinical isolates identified by other molecular methods (Baele *et al.*, 2000; Dijkshoorn *et al.*, 1998; Nemec *et al.*, 2001; Vaneechoutte *et al.*, 1998) were used for sensitivity testing of the array, in order to avoid unnecessary discrepant identifications that should be reconfirmed by other molecular techniques. Of 153 target clinical isolates tested, only one (*Moraxella caviae* #003) was not identified by the array (Table 3). However, the identity of this strain as *M. caviae* could not be confirmed by either ITS or 16S rRNA gene sequencing. If *M. caviae* #003 was excluded for sensitivity calculation and if reference strains (123 strains) and clinical isolates (152 strains) were taken together, the sensitivity of the array was 100 % (275/275).

In addition, a collection of 128 clinical isolates, identified by API 20 NE as belonging to one of the 38 target species, were also analysed by the array (data not shown). Of these, 12 produced discrepant identifications between API 20 NE and the array. Eleven of the 12 discrepant isolates were correctly identified by array hybridization, as further confirmed by both ITS and 16S rRNA gene sequencing. One discrepant strain (5630A) was not identified by the array, and both ITS and 16S rRNA gene sequence analyses revealed that the strain was *Bordetella hinzii*, which was not a target species on the array (data not shown). The results again highlight the accuracy of the array and the inaccuracy of biochemical tests for identification of non-fermenters.

Specificity of the array

A collection of 122 non-target strains (106 species) were used for specificity testing of the array (see Supplementary Table S1). A total of four strains were misidentified. *Burkholderia cenocepacia* BCRC 17448^T was misidentified as *Burkholderia cepacia* (Fig. 2, chip 48), *Chryseobacterium gleum* BCRC 17270 was misidentified as *Chryseobacterium indologenes*, and *Shewanella algae* LMG 2267 and #017 (a clinical isolate) were misidentified as *Shewanella putrefaciens* by the array, resulting in a specificity of 96.7 % (118/122) for the array. Sequence analysis of the 16S rRNA gene revealed that the identities of the four cross-hybridization strains were correct (data not shown).

In this study, most individual species were identified by a single oligonucleotide probe, but multiple probes were used to identify *A. junii*, *A. lwoffii*, *P. stutzeri* and *Shewanella putrefaciens* (Table 2). The disadvantage of using multiple probes is the increased potential of cross-hybridization caused by other species that may have partial sequence similarity with one of the multiple probes. *P. stutzeri* has been reported to be involved in a variety of severe infections including bacteraemia, endocarditis and meningitis (Noble & Overman, 1994). Bacteria identified as *P. stutzeri*, on the basis of phenotypic tests, are recognized to be very heterogeneous and can be divided into nine genomovars based on DNA similarities (Guasp *et al.*, 2000). Sequencing of the ITS region was proposed as a good alternative for genomovar differentiation of species of the *P. stutzeri* complex (Guasp *et al.*, 2000). Due to the heterogeneity of the complex, it was difficult to find a consensus region that covered all genomovars of the micro-organism and consequently four probes were designed to identify the bacterium (Table 2).

The successful design of different probes was based on known sequences in the ITS regions and multiple sequence alignment played an important role in pinpointing the regions that could be used for probe synthesis. It should be noted that some non-fermenters (e.g. *Acinetobacter haemolyticus* and *A. lwoffii*) possess multiple ITS regions of different length and sequence (Chang *et al.*, 2005). Oligonucleotide probes targeting any of these ITS regions can be used for identification.

In conclusion, an oligonucleotide array was developed to identify 38 species of Gram-negative non-fermenters. With a sensitivity of 100 % and a specificity of 96.7 %, this array provides a rapid and relatively accurate method for species identification of clinically relevant non-fermenters. The results of array hybridization are available within one working day.

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