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ORIGINAL ARTICLE

Evidence of the adaptive response in *Pseudomonas aeruginosa* to 14 years of incubation in seawater

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Abstract The adaptive response of bacteria to stress is variable owing to the effects of various environmental factors and the time of exposure to stress conditions. We have studied the morphological and biochemical characters of resuscitated *Pseudomonas aeruginosa* ATCC 27853 that had been incubated for 14 years in natural sterilized seawater. The resuscitated cells were identified by PCR analysis of the internal transcribed spacer region. The biochemical profile of the resuscitated cells was partially inactive according to the API-20NE system. The cells had lost the ability to reduce nitrate and hydrolyse gelatin but acquired urease activity. In addition, the atomic force micrographs showed a reduction in cell size and an evolution to coccoid shapes. After incubation in a rich medium, the resuscitated cells regained their initial biochemical characters and their original rod shape. The differential display "DDRT-PCR" technique was used to determine the effects of stress on gene expression in this *P. aeruginosa* strain. The genes *algU* and *iscR* were successfully identified in stressed *P. aeruginosa* ATCC 27853 as two differentially expressed gene fragments. Our results demonstrate that the DDRT-PCR technique can be a useful tool in gene expression analysis studies looking at the stress response in *P. aeruginosa*.

Keywords *Pseudomonas aeruginosa* · Seawater · Resuscitation · Atomic force microscopy · DDRT-PCR

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Introduction

Pseudomonas aeruginosa is a metabolically versatile Gram-negative bacterium that inhabits terrestrial and aquatic environments (Wiehlmann et al. 2006). It is also found regularly on the surfaces of plants and occasionally on the surfaces of animals, where it exploits injuries to the host to initiate an infection (Rodríguez-Rojas and Blazquez 2009). *P. aeruginosa* is a typical representative of opportunistic human pathogens, being implicated in a wide range of infections of the pulmonary tract, urinary tract, burns and wounds, among others (Balasubramanian and Mathee 2009). In addition, it causes persistent nosocomial infections and is also a major pathogen in cystic fibrosis patients.

Prokaryotes may survive inside fluid inclusions for tens of thousands of years using carbon and other metabolites supplied by the trapped microbial community. The most notable example of this is the single-celled alga *Dunaliella*, an important primary producer in hypersaline systems (Lowenstein et al. 2011). The capacity of different non-halophytic bacterial species to survive for long periods of time into seawater has also been documented (Bakhrouf et al. 2008; Dhiaf et al. 2010). *P. aeruginosa* has a high metabolic versatility, including the ability to adapt to virtually all aquatic mesopholic habitats, such as seawater (Kiewitz et al. 2000). A number of recent studies have documented the existence and survival of *P. aeruginosa* in the open ocean (Kimata et al. 2004; Khan et al. 2007). Even more recently, Khan et al. (2010) reported that the marine strain of *P. aeruginosa* grew better in microcosms containing a high level of NaCl (7% w/v) whereas the freshwater strain did better at 0–3% NaCl. On the other hand, *P. aeruginosa* PAO1 strain was found to be able to tolerate 700 mM NaCl (Velasco et al. 1995). It has also been documented that the number of culturable cells of *P. aeruginosa* incubated in

sterile nutrient-free seawater decreased progressively over time and that the bacterial cells that developed were capable of passing through a 0.45-μm pore membrane due to a reduction in size; it was also found to be able to modify some biochemical characters, such as gelatinase or urease activity (Bakhrouf et al. 1989).

Pseudomonas aeruginosa subjected to osmotic stress in the form of high NaCl concentrations is able to induce a group of genes involved in its adaptation to this kind of stress. In fact, it has been shown to be able to adapt to environmental stress by inducing specific sets of genes (Jorgensen et al. 1999). Moreover, a large number of differences in the expression pattern have been observed between steady state osmotic stress and the osmotic up-shock after 15 and 60 min in *P. aeruginosa*. These differences demonstrate the importance of the incubation period in a high salt concentration on the adaptive response, especially on gene expression (Aspedon et al. 2006).

Analysis of gene expression is a central aim in most studies focusing on understanding the adaptive response of bacteria to stress conditions. Interest in stress-specific gene expression has stimulated the search for proper methods to identify the actual differences between the normal situation and the stressed one. Several molecular strategies, including microarray analysis (Termine et al. 2009), in vivo expression technology (Ramos-Gonzalez et al. 2005), differential display reverse transcription-polymerase chain reaction (DDRT-PCR) (Fleming et al. 1998; Gill et al. 1999), signature-tagged mutagenesis (Handfield and Levesque 1999) and suppression subtractive hybridization (De Long et al. 2008) have been used to identify stress-associated genes in bacteria under different growth and environmental conditions. Although DDRT-PCR was originally described for the analysis of eukaryotic systems, some modifications have allowed this technique to be used in prokaryotes (Fleming et al. 1998). In addition, DDRT-PCR has several advantages, including the need for only small amounts of RNA and the lack of a requirement for specifically designed genetic tools (Park et al. 2004).

The aim of this study was to investigate the biochemical and morphological variations in resuscitated *P. aeruginosa* ATCC 27853 cells that had been incubated for 14 years in natural sterile seawater. The DDRT-PCR method was used to investigate the transcriptional response of *P. aeruginosa* to stress conditions.

Experimental

Bacterial strain and growth conditions

Pseudomonas aeruginosa ATCC 27853 was grown overnight at 37°C in nutrient broth, centrifuged at 13,000 rpm for 10 min, washed three times and then suspended in 100-ml filtered sterilized seawater to a final concentration of approximately 10^9 CFU/ml, according to the method described by Ben Abdallah et al. (2008). The microcosm was then incubated for 14 years in a closed Erlenmeyer flask at room temperature.

Resuscitation of viable but non-culturable cells

Stressed cells of *P. aeruginosa*, incubated for 14 years in sterilized seawater, were resuscitated by the addition of autoclaved nutrient broth and cultured in nutrient agar. The biochemical activities of *P. aeruginosa* cells were characterized using the API-20NE system (bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

Determination of morphological changes by atomic force microscopy

The stressed *P. aeruginosa* cells were examined by atomic force microscopy (AFM) to visualize the morphological changes (Nanoscope IIIA; Digital Instruments/Veeco, Santa Barbara, CA). The cells were collected, washed three times with phosphate-buffered saline (PBS), and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and air dried. Three independent cells of the same sample were measured. An unpaired Student's t test was used for statistical analysis. P values ≤ 0.05 were considered to be statistically significant.

Molecular confirmation of resuscitated strain

A PCR analysis was performed according to Tyler et al. (1995 to confirm the identity of the starved cells of *P. aeruginosa* obtained after resuscitation. Bacteria were first cultured on Luria–Bertani agar medium for 24 h at 37°C. One colony was then cultured in nutrient broth for 24 h at 37°C, and 1 ml of the culture was centrifuged. The DNA was extracted by boiling for 5 min at 100°C, incubation on ice for 5 min and centrifugation at 13,000 rpm for 8 min. The supernatant was used for PCR amplification.

PCR was performed in a 25-μl reaction volume containing 50 ng of extracted DNA, 5 μl 5× PCR buffer with 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphates (dNTPs; JenaBioscience, Jena, Germany), 1 μM of each internal transcribed spacer (ITS) primer (forward: 5′-TCCAAACAATCGTCGAAAGC-3′; reverse: 5′-CCGAAAATTCGCGCTTGAAC -3′) and 1 U of Taq DNA polymerase (Biotools B&M Labs, Madrid, Spain). Amplification was conducted in a MultiGene gradient thermal cycler (Labnet International, Edison, NJ). The

reaction mixtures were heated at 94°C for 2 min and then subjected to 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min, followed by a 10-min final extension period at 72°C. The PCR products (5 μl) were analysed by electrophoresis (1% agarose gel, 100 V, 30 min), and the products stained with ethidium bromide (0.5 mg/ml) and visualized under ultraviolet transillumination. The amplification products were photographed and their sizes determined by comparison with a 100-bp molecular size marker.

Table 1 Sequences of the arbitrary primers used in differential display-PCR reactions

Primer	Sequence 5′–3′
AP17	AAGCTTACCAGGT
AP18	AAGCTTAGAGGCA
AP19	AAGCTTATCGCTC
AP20	AAGCTTGTTGTGC
AP21	AAGCTTTCTCTGG
AP22	AAGCTTTTGATCC
AP23	AAGCTTGGCTATG
AP24	AAGCTTCACTAGC

Isolation of RNA and reverse transcription

For total RNA extraction, the wild-type *P. aeruginosa* ATCC 27853 and its stressed strain obtained after 48 h of resuscitation were cultivated overnight in DM medium prepared with filtered sterilized seawater (6.78 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.28% glucose, 0.25% casamino acids) at 37°C and 200 rpm. Total RNA was isolated using the SV total RNA isolation system, following the manufacturer's instructions (Promega, Madison, WI). The RNA was digested with DNase I to eliminate residual DNA using the Message Clean kit (GenHunter, Nashville, TN). RNA was electrophoresed in a formaldehyde agarose gel to check the integrity of the samples.

For the reverse transcription (RT) reactions, a mixture of 1 μg of RNA, 1 μl of random hexamers (3 μg/μl; Invitrogen, Carlsbad, CA), 2 μl of 10 mM dNTPs (Jena Bioscience), 4 μl of 5× first-strand buffer (250 mM Tris–HCl at pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2.0 μl of 0.1 M dithiothreitol and 100 units of Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Promega) was brought up to a final volume of 20 μl with deionized water (dH_2O) treated with diethylpyrocarbonate. The synthesis reaction was carried out for 60 min at 40°C in a MultiGene gradient thermal cycler (Labnet International) and stopped by incubation at 75°C for 10 min. To avoid detecting DNA contamination in DDRT-PCR, we used one control for each RNA sample without MMLV reverse transcriptase.

Differential-display PCR

The cDNAs were amplified under relatively relaxed conditions as described by Hill et al. (2003). The 20-μl total reaction volume contained 6 μM $MgCl_2$, 0.8 μl of RT reaction, 1.5 μM of arbitrary 13-mer primers (Table 1), 0.15 mM dNTPs, 2 μl of 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl) and 2.5 units of Taq DNA polymerase (Biotools B&M Labs). The amplification conditions comprised 5 min at 94°C, 40 cycles of 1 min at 94°C, 2 min at 40°C and 30 s at 72°C, and a final 5-min elongation at 72°C. Arbitrary primers were obtained from the GenHunter Corp. (Nashville, TN). Twenty primer pairs were tested for their ability to amplify *P. aeruginosa* cDNAs. For each combination of 13-Mer arbitrary primer, the reaction was performed in triplicate. The DD-PCR products were electrophoresed in a 2% agarose gel in 1× TBE buffer (0.089 M Tris-acetate, 0.089 M boric acid, 0.002 M ethylene diamine tetraacetic acid, pH 8.3) and stained with ethidium bromide.

Identification of the differentially expressed cDNA fragments

To identify the differentially expressed genes in stressed *P. aeruginosa*, we cut out the bands of interest from an agarose gel with a sterile razor blade and then purified these using the EZ-10 Spin Column DNA Gel Extraction kit (BIO BASIC, Amherst NY) according to the manufacturer's instructions. Re-amplification of the cDNA fragments was carried out under the same conditions as those for the differential-display PCR step. DNA sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA) using the ABI BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). DNA sequence homology search within the GenBank database was performed using BLAST.

Results

Resuscitation of VBNC cells

Pseudomonas aeruginosa that had survived for 14 years in natural sterile seawater could be cultivated on nutrient agar after a 24-h incubation in nutrient broth. The biochemical profile of these cells was partially inactive on the API-20NE system. The cells could not perform nitrate reduction nor gelatine hydrolysis, but they had acquired urease activity. The resuscitated cells regained their initial bio-

chemical characters after 48 h of incubation in nutrient broth (Table 2).

Molecular confirmation of resuscitated strain

After 24 h of resuscitation, *P. aeruginosa* became unidentifiable by the API-20NE system. We therefore used the PCR technique to identify this microbe. The ITS region, which corresponds to the region between the 16S and 23S rRNA gene, is a preserved region in bacteria with a characteristic length in *P. aeruginosa* species (181 bp). After amplification of the ITS region by PCR, we confirmed the identity of the resuscitated bacteria that had been incubated for 14 years in sterile seawater (Fig. 1).

Table 2 Evolution of biochemical characters following the resuscitation of *Pseudomonas aeruginosa* ATCC 27853 incubated for 14 years in natural sterile seawater

Enzyme	SBI[a]	Strain after resuscitation	
		24 h	48 h
NO_3	+	(–)	+
TRP	-	-	-
GlU	-	-	-
ADH	+	+	+
URE	-	(+)	-
ESC	-	-	-
GEL	+	(–)	+
PNPG	-	-	-
GLU	+	+	+
ARA	-	-	-
MNE	-	-	-
MAN	+	+	+
NAG	+	+	+
MAL	-	-	-
GNT	+	+	+
CAP	+	+	+
ADI	+	+	+
MLT	+	+	+
CIT	+	+	+
PAC	-	-	-
OX	+	+	+

+, Presence of character; -, absence of character; ( ), altered character

NO_3, Nitrate reduction; TRP, indole production; GlU, glucose acidification; ADH, arginine dihydrolase; URE, urease; ESC, esculin hydrolysis; GEL, gelatin hydrolysis ; PNPG, *p*-nitropyhenyl-ßD galactopyranoside; GLU, glucose assimilation; ARA, arabinose assimilation; MNE, mannose assimilation; MAN, mannitol assimilation; NAG, N-acetyl glucosamine; MAL, maltose glucosamine; GNT, gluconate glucosamine; CAP, caprate glucosamine; ADI, adipate glucosamine; MLT, malate glucosamine; CIT, citrate glucosamine; PAC, phenyl acetate glucosamine; OX, cytochrome oxidase

[a] SBI, strain before incubation in seawater

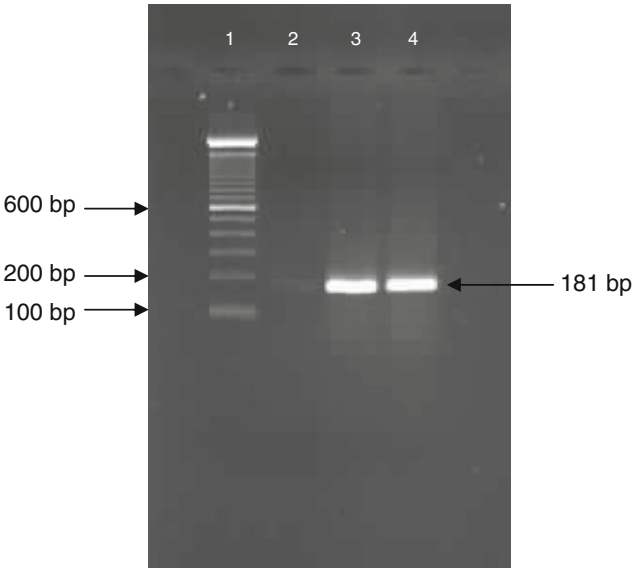

Fig. 1 PCR products amplified from the internal transcribed spacer region of the studied *Pseudomonas aeruginosa* strains. *Lanes*: *1* 100-bp molecular size marker, *2* negative control, *3* non-stressed *P. aeruginosa* ATCC 27853 (positive control), *4 P. aeruginosa* ATCC 27853 strain incubated 14 years in seawater

Morphological variations of stressed strain

The morphological alterations were examined by AFM (Fig. 2). The control *P. aeruginosa* ATCC 27853 cells had a length of about 2 μm (1.83 μm±0.20) and have a normal rod shape (Fig. 2, A1, B1). In contrast, the cells obtained after 24 and 48 h of resuscitation had a coccoid shape and were <1 μm long (0.55 μm±0.15) (Fig. 2, A2, B2). After 2 months of incubation in nutrient broth, the cells had regained their original size (1.76 μm±0.25) and shape (rod) (Fig. 2, A3, B3).

Differential display of *P. aeruginosa* gene expression

In order to determine the effects of salinity on gene expression in *P. aeruginosa*, we analysed RNA by the DDRT-PCR technique. Total RNA extracted from the wild-type *P. aeruginosa* ATCC 27853 and its stressed strain was of good quality, with no detectable degradation of rRNA by gel electrophoresis, as shown in Fig. 3. The first cDNA strand was obtained using random hexamers as primers. These short primers provided cDNA for a larger number of different genes. We compared cDNA from control *P. aeruginosa* ATCC 27853 and its stressed strain obtained after 48 h of resuscitation by RT-PCR using arbitrary primers. The reaction pairs of each combination of arbitrary primers were run in adjacent wells in a 2% agarose gel (Fig. 4). The number of DDRT-PCR products in each electrophoretic profile ranged between 0 and 16 depending on the primer pair used. This analysis clearly demonstrated

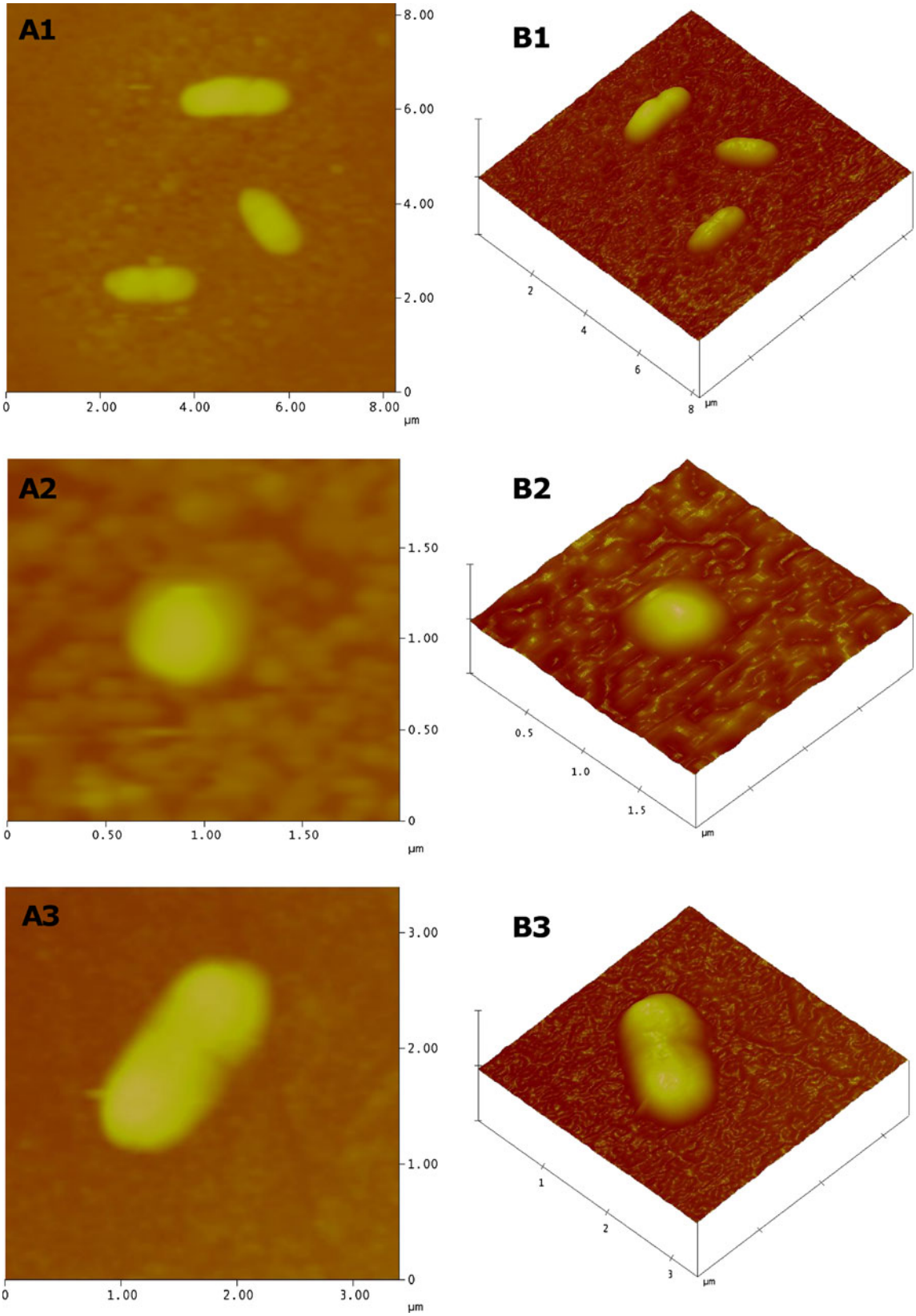

Fig. 2 Two-dimensional analysis and three-dimensional profiles of atomic force micrographs of resuscitated *P. aeruginosa* cells. **A1**, **B1** cells prior to incubation in seawater, **A2**, **B2** cells obtained after 24 h of resuscitation, **A3**, **B3** cells obtained after 2 months of resuscitation

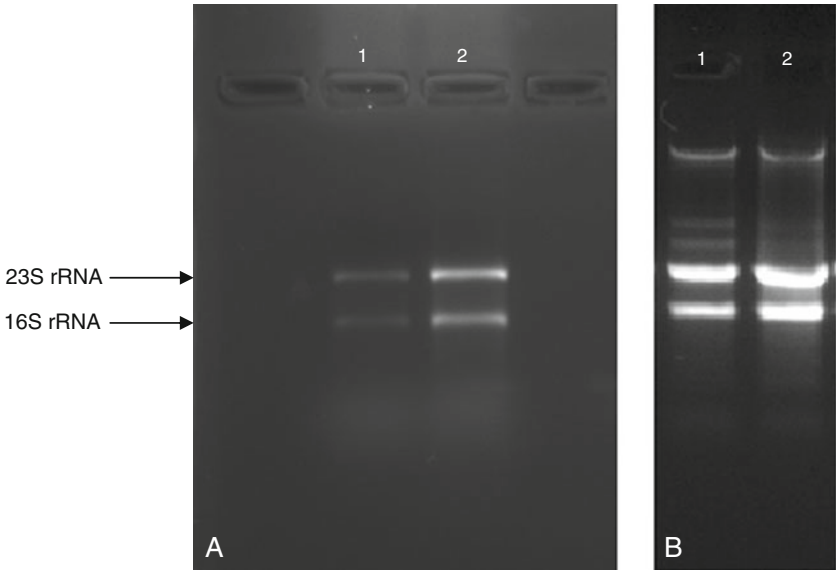

Fig. 3 Results of formaldehyde 1% agarose gel for RNA quality verification. **a** RNA samples after DNase I treatment; *lanes*: 1 stressed *P. aeruginosa* ATCC 27853 strain, *2* control strain. **b** RNA samples before DNase I treatment; *lanes* 1 stressed *P. aeruginosa* ATCC 27853 strain, *2* control strain

that the genes analysed were differentially expressed, as evidenced by changes in band appearance or disappearance. We compared the same arbitrary primer combination products of the stressed and wild-type strains side by side and selected not only the bands present in the stressed sample and absent in the control one, but also those bands with a higher intensity under salinity conditions compared with standard ones (Fig. 4). The number of genes upregulated during stress was always higher than the number of genes downregulated.

Identification of differentially expressed genes

To obtain more information on the kind of regulated genes present in stressed *P. aeruginosa*, we identified two selected cDNA fragments in *P. aeruginosa* ATCC 27853 incubated for 14 years in seawater. Based on the deduced amino acid sequences two upregulated genes were homologues to *iscR* (iron-sulfur cluster assembly transcription factor) and *algU* (transcription regulator, sigma factor). The percentage of amino acid identity of the differentially expressed genes is shown in Table 3.

The percentage nucleotide sequence identity between fragment F1 and the *algU* gene for four strains of *P. aeruginosa* was 100%, proving the identification of the cDNA fragment and its conservation.

The homology between the nucleotide sequence of F2 fragment was found to be highly conserved to the *iscR* gene among the two reference strains: *P. aeruginosa* PAO1 (99%) and *P. aeruginosa* PA7 (95%) (Table 3). However, when the F2 sequence was compared with *iscR* of *P. aeruginosa* PA7, we found eight nucleotide differences, of which only one was detected in the *P. aeruginosa* PAO1 *iscR* sequence (Fig. 5). Despite, this slight nucleotide difference, we did not observe any change in the amino acid sequences. The high conservation of the *IscR* and *algU* amino acid sequence demonstrates the major role of both genes in adapting to stress conditions.

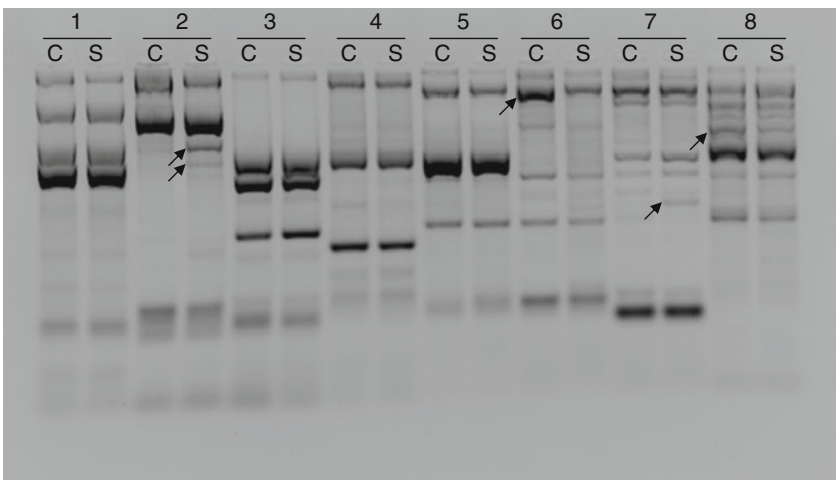

Fig. 4 Examples of differential display reverse transcription-PCR results obtained using 2% agarose gel for *P. aeruginosa* cells. *Lanes*: *S* Stressed strain, *C* control strain. *Numbers* Pairs of arbitrary primers combinations used for differential display-PCR (*1* AP19 + AP20, *2* AP23 + AP24, *3* AP20 + AP24, *4* AP19 + AP21, *5* AP18 + AP22, *6* AP18 + AP21, *7* AP18 + AP20, *8* AP18 + AP19). *Arrows* indicate examples of differentially displayed bands

Table 3 Preliminary BLAST alignments of differentially and constitutively expressed transcripts

Fragment name	Size (bp)	Identified gene	Description/Function	Organism	Accession number	Homology	Amino acid identity
F1	232	*algU*	Transcription regulator activity, sigma factor	*P. aeruginosa* PAO581	EF635219	100%	100%
				P. aeruginosa C3715c	EF540908	100%	100%
				P. aeruginosa C0686c	EF540907	100%	100%
				P. aeruginosa PAO1	EF540906/PA0762	100%	100%
F2	221	*iscR*	Iron-sulfur cluster assembly transcription factor IscR, Regulatory function	*P. aeruginosa* PAO1	PA3815/AE004091	99% (220/221)	100%
				P. aeruginosa PA7	PSPA7_1298/ CP000744	96% (213/221)	100%

Nucleotide sequence accession numbers

Sequence data of the identified cDNA have been deposited in the EMBL sequence database under accession numbers HE599788 (F1 fragment: *algU* gene) and HE599789 (F2 fragment: *iscR* gene.

Discussion

The results of this study show that *P. aeruginosa*, a ubiquitous microbe, can transform into a viable but non-culturable (VBNC) state with biochemical and morphological modifications when faced with stressing environmental

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             1                          *                        50
    F2-iscR  ACGCGGTCAA CGAATCCGTC GACGCGACCC GCTGCCAGGG GCAGGGCGAT
     PA3815  ACGCGGTCAA CGAATCCGTC GACGCGACCC GCTGCCAGGG GCAGGGCGAT
 PSPA7_1298  ACGCGGTCAA CGAATCCGTC GACGCCACCC GCTGCCAGGG GCAGGGCGAT
  Consensus  ACGCGGTCAA CGAATCCGTC GACGCgACCC GCTGCCAGGG GCAGGGCGAT

             51   *      *         *  *                         100
    F2-iscR  TGCCACTCCG GCGATACCTG TCTGACCCAC CACCTGTGGT GCGACCTCAG
     PA3815  TGCCACTCCG GCGATACCTG TCTGACCCAC CACCTGTGGT GCGACCTCAG
 PSPA7_1298  TGCCATTCCG GTGATACCTG CCTCACCCAC CACCTGTGGT GCGACCTCAG
  Consensus  TGCCAcTCCG GcGATACCTG tCTgACCCAC CACCTGTGGT GCGACCTCAG

             101      *                *                        150
    F2-iscR  TTTGCAGATC CATGAGTTCC TCAGCGGCAT CAGCCTGGCC GATCTGGTCA
     PA3815  TTTGCAGATT CATGAGTTCC TCAGCGGCAT CAGCCTGGCC GATCTGGTCA
 PSPA7_1298  TTTGCAGATC CATGAGTTCC TCAGTGGCAT CAGCCTGGCC GATCTGGTCA
  Consensus  TTTGCAGATc CATGAGTTCC TCAGcGGCAT CAGCCTGGCC GATCTGGTCA

             151                 *      *                        200
    F2-iscR  GCCGCCAGGA GGTCCAGGAG GTCGCGCTGC GCCAGGACGA ACGCCGTTGC
     PA3815  GCCGCCAGGA GGTCCAGGAG GTCGCGCTGC GCCAGGACGA ACGCCGTTGC
 PSPA7_1298  GCCGCCAGGA GGTCCAGGAA GTCGCACTGC GCCAGGACGA ACGCCGTTGC
  Consensus  GCCGCCAGGA GGTCCAGGAg GTCGCgCTGC GCCAGGACGA ACGCCGTTGC

             201                 221
    F2-iscR  TCAGGCAAGA CGCCTCGTCT C
     PA3815  TCAGGCAAGA CGCCTCGTCT C
 PSPA7_1298  TCAGGCAAGA CGCCTCGTCT C
  Consensus  TCAGGCAAGA CGCCTCGTCT C
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Fig. 5 Partial nucleotide sequence alignment of the *iscR* gene. *F2-iscR* Nucleotide sequence of the identified cDNA fragment of *P. aeruginosa* ATCC 27853 (determined in this study; accession number HE599789). *PA3815* Nucleotide sequence of the *iscR* gene of *P. aeruginosa* PAO1 (from GeneBank). *PSPA7-1298* Nucleotide sequence of *iscR* gene of *P. aeruginosa* PA7 (from GeneBank). *Asterisk* No conserved nucleotides

conditions—in our study, 14 years of incubation in seawater. The resuscitated cells had a coccoid shape and had lost nitrate reduction and gelatin hydrolysis activity, but they had acquired urease activity. According to Bakhrouf et al. (1989), *P. aeruginosa*, when incubated in sterile nutrient-free seawater, is able to modify some of its biochemical characters, such as gelatinase or urease activity. However, our results demonstrate that VBNC cells of *P. aeruginosa* can be resuscitated and that they regain their initial characters when favourable growth conditions are restored. VBNC cells of *P. aeruginosa* ATCC 27853 incubated for 14 years in seawater were resuscitated after a 24-h incubation in nutrient broth. Similarly, *Salmonella enterica* serovar Agona, incubated for 13 years in soil, returned to a cultivable state on tryptic soy agar after a 24-h incubation in tryptic soy broth (Bakhrouf et al. 2008). Recently, VBNC 20-year-starved *Salmonella enterica* serovar Typhimurium, in seawater and soil, was recovered after a 2-month incubation in nutrient broth (Dhiaf et al. 2010).

The long survival (14 years) of *P. aeruginosa* in natural sterile seawater was accompanied by morphological modifications, including a reduction in cell size and transformation to a coccoid shape. The reduction in cell size is the result of cytoplasmic contraction as well as a reduction in bacterial periplasm volume (Huisman et al. 1996). During this size reduction, significant changes in membrane structure, protein composition, ribosomal content and possibly even DNA arrangement occur (Oliver 2000). Indeed, reducing cell size and, consequently, minimizing the need for nutrients, is one strategy by which bacteria adapt and survive stress conditions (Jiang and Chai 1996). The evolution towards this state can enable the bacterium to survive for a long period under starvation conditions. One proposed mechanism for coccoid cell formation may, in fact, represent an adaptation of *P. aeruginosa* and other Gram-negative bacteria to constantly changing environmental conditions (Coutard et al. 2007).

Pseudomonas aeruginosa has become an important cause of serious infection, especially in immunocompromised or otherwise susceptible hosts. It causes serious and often fatal opportunistic infections, ranging from systemic acute disease in burned and neutropenic patients to chronic infections of the respiratory tract in cystic fibrosis patients (Yu et al. 1996). Pseudomonal infections are complicated and can be life threatening. This pathogen is widespread in various aquatic environments, and it can persist and survive under extreme conditions, entering into the VBNC state. The identification of such stressed cells by routine laboratory methods is difficult once the defining morphological and/or biochemical characters have been lost, and the 'hidden' pathogen can induce various health complications. Consequently, molecular biology methods, such as DDRT-PCR or micoarray, are needed to identify VBNC cells in general and, more specifically, to characterize the adaptive response of *P. aeruginosa* to long-term incubation in hostile environments. Such molecular tools will facilitate exploration of the expression of virulence factors in these pathogens. Stable virulence of other bacterial species in the VBNC state has been reported; (Macro–Noales et al. 1999) demonstrated that starved *Vibrio vulnificus* cells were able to retain their ability to infect eels and humans and that they retained their virulence at a level similar to that of the nonstarved cells. These authors also demonstrated that after 2 years of starvation, *V. vulnificus* cells from saline microcosms were able to infect eels and mice. Ben Kahla et al. (2008) found that *Vibrio fluvialis* cultured in a in seawater microcosm for a long period of time also maintained its virulence.

Pseudomonas aeruginosa responds to changing environmental conditions by using a number of adaptive survival mechanisms, including a change in the pattern of gene expression. The results of our DDRT-PCR analysis demonstrate the great potential of mRNA differential display methodologies to unravel differential gene expression in cells exposed to stress conditions. Indeed, the DDRT-PCR profiles in our study showed the presence of specific bands linked to long-term salt stress. To understand how *P. aeruginosa* survives in osmotically stressful environments for a long time (many years), we need to know which genes are differentially regulated following exposure to stress. It was initially expected that the differentially detected bands obtained in the DDRT-PCR profiles would be associated with osmoprotective genes and regulatory factors controlling their expression.

Based on the deduced amino acid sequences and the BLAST analysis, we identified two upregulated genes in stressed *P. aeruginosa* ATCC 27853. *iscR*, which encodes an iron-sulfur cluster assembly regulator homologue, is one of the genes required for peroxide resistance in *P. aeruginosa* and it is related to the proper activity of KatA, which is crucial for peroxide resistance and full virulence of this bacterium (Kim et al. 2009). The IscR protein differentially regulates the expression of a large number of genes under aerobic and anaerobic conditions (Giel et al. 2006). It has also been shown recently that IscR regulates biofilm formation in *Escherichia coli* (Wu and Outten 2009). As the formation of biofilm can limit iron availability and/or stress bacteria, it has been suggested that IscR responds to stresses with regulatory function (Fleischhacker and Kiley 2011).

The *algU* gene encodes an alternative sigma factor which is a founding member of stress-responsive alternative sigma factors. It has been implicated in the activation of defence systems necessary for cell survival under extreme conditions such as high temperature or exposure to reactive oxygen intermediates (Yu et al. 1996). It is therefore

possible that AlgU (sigmaE), in addition to regulating alginate production, may affect the expression of additional stress response systems of possible relevance for pathogenesis. AlgU plays a central role in virulence that is linked to its capacity to protect the bacterium from lethal host defences. Results from in vitro experiments suggest that the basis for this protection lies in the ability of sigmaE to induce resistance to a variety of envelope stresses (Raivio 2005). Furthermore, the sigmaE response appears to be predominantly involved in survival within the host at post-invasion steps of disease.

Similarly, using DDRT-PCR, Holmstrom and Gram (2003) demonstrated that during the stress response, the fish pathogen *Vibrio anguillarum* expresses a gene homologous to *rpoS*. The response of *P. aeruginosa* under starvation conditions is due to the expression of certain genes whose regulation is controlled by the RpoS protein and other transcriptional factors (Jorgensen et al. 1999). Additionally, *rpoS*, which encodes sigma factors in *E. coli*, are involved in morphological alteration of the cell after transition to the stationary phase (Lange and Hengge-Aronis 1991). DDRT-PCR analysis has also enabled the isolation and identification of 32 *Listeria monocytogenes* gene fragments that were observed to be more highly expressed under cabbage-associated conditions (Palumbo et al. 2005).

Genes differentially expressed in response to osmotic stress may play a key role in the prevalence and persistence of *P. aeruginosa* (Govan and Deretic 1996). Using microarray analysis, Aspedon et al. (2006) demonstrated that many differences in the gene expression patterns of *P. aeruginosa* were observed between steady state osmotic stress and the osmotic up-shock after 15 and 60 min of osmotic stress. In addition, a large number of differentially regulated open reading frames with unknown functions were detected, demonstrating the importance of the length of the incubation in high salt concentrations on transcriptional regulation. Moreover, the differential expression of some hypothetical proteins highlights the fact that many of the response mechanisms of *P. aeruginosa* to osmotic stress are uncharacterized. The potential role of many of these proteins on stress adaptation is currently being investigated, and the results of these studies will broaden our perspectives towards characterizing novel genes involved in osmotic stress response of *P. aeruginosa*.

DDRT-PCR can be used and applied in research on organisms, particularly to study transcript profiling, for which microarray analysis is not available or not economically feasible (Venkatesh et al. 2005). Although one of the major problems in analysing differences in gene expression in bacteria derives from the fact that mRNA in prokaryotes does not have a polyA tail or has a low rate of polyadenylated mRNA (Saravanamuthu et al. 2004), we describe here the success of our DDRT-PCR analysis in determining *P. aeruginosa* gene regulation in response to long-term stress and in identifying two differentially regulated genes implicated in the adaptive response of *P. aeruginosa*. Differential hybridization has long been the only method enabling the isolation of genes in these two contexts (Bauer et al. 1994). We suggest that DDRT-PCR can become one of the most widely available techniques for the identification of differences in gene expression in bacterial species. It offers a rapid and simultaneous comparison of the expression profiles of mRNA from cells which have adapted to different stress situations. This technique has a number of advantages over differential hybridization, including the simultaneous display of all differences and the simultaneous detection of the upregulation and down-regulation of genes; it is also faster (Bauer et al. 1994).

Given that our the DDRT-PCR analysis was performed in triplicate in order to detect differentially expressed genes, another suitable method, such as real-time PCR, is required to confirm the actual differential regulation. Interestingly, similar to other microorganisms (Ben Kahla et al. 2008; Bakhrouf et al. 2008), our results confirm that *P. aeruginosa* is capable of adapting and surviving a long incubation period in sea water (14 years). Also, these findings suggest that specific mechanisms of adaptation should be studied and that the network of regulatory genes in the context of transcriptomics and genetics should be traced.

In conclusion, *P. aeruginosa* responds to long-term salt conditions by using a number of adaptive survival mechanisms. These mechanisms include a change in the pattern of gene expression, often manifested physiologically, biochemically and structurally. The understanding of the adaptive response of the opportunistic human pathogen *P. aeruginosa* in long-term stressful conditions may be of great interest to researchers working in the fields of clinical and environmental microbiology.

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