Phenotypic stress response of *Pseudomonas aeruginosa* following culture in water microcosms

Jihane Cheriaa, Mahmoud Rouabhia, Makaoui Maatallah and Amina Bakhrouf

ABSTRACT

The purpose of the present study was to explore the potential behavioural changes of *Pseudomonas aeruginosa* following growth in different aquatic environmental conditions. To achieve this, *P. aeruginosa* was cultured in various water microcosms for 12 months under fixed (pH, nutrients and temperature) factors. *P. aeruginosa* responses to these conditions were investigated using colony morphotype, biochemical and enzymatic characterisation, pyocin typing, serotyping, sensitivity to different classes of antibiotics and molecular identification. Results show that starvation in water microcosms lead to unusual phenotypes. Of interest is that the pyocin changed from 24/n in the wild type to 83/a following culture in the water microcosms, and the serotype changed from O6 in the wild type to O1 in microcosm-cultured *P. aeruginosa*. Furthermore, the starvation period in various aquatic microcosms enhanced the resistance of *P. aeruginosa* against beta-lactam antibiotics. Compared to the other aquatic environments, the seawater microcosm produced the greatest amount of variations in *P. aeruginosa*. Overall, data demonstrated a high adaptability of *P. aeruginosa* to environmental changes. This may explain the unusual antibiotic-resistant phenotypes belonging to *P. aeruginosa* species, and their capacity for spreading that leads to human infections.

Key words | antibiotic resistance, pyocin typing, serotyping, starvation, 16S–23S rDNA intergenic transcribed spacer (ITS)

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterial pathogen involved in a broad range of infections. It is responsible for chronic lung infections complicating cystic fibrosis (CF). In persons who have CF, acquisition of *P. aeruginosa* is associated with a decline in pulmonary function, and increased morbidity and mortality (Hauser *et al.* 2011). Infection with *P. aeruginosa* can also occur in individuals without CF. Examples include keratitis, and infections of the skin (burn superinfections and folliculitis), urinary tract, upper and lower respiratory tract (tracheobronchitis and pneumonia) and bloodstream (Zilberberg *et al.* 2010; Aujoulat *et al.* 2011; Deguchi *et al.* 2011). Compromise of epithelial barrier function and loss of local immune defences predisposes individuals to *P. aeruginosa* infections. These important barriers to opportunistic infection are often breached iatrogenically, through urinary tract catheterisation, tracheal intubation and mechanical ventilation. Indeed, *P. aeruginosa* is one of the more common causes of intensive care unit infections and is among the leading causes of ventilator-associated pneumonia (Venier *et al.* 2011). The intrinsic resistance of *P. aeruginosa* to antibiotics also makes it difficult to eradicate from equipment and surfaces in healthcare facilities and hospitals (Master *et al.* 2011).

P. aeruginosa has a remarkable ability to adapt and thrive in a variety of environments, including seawater (Kimata *et al.* 2004), soil (Cavalca *et al.* 2000) and industrial water, effluents and other fluids, such as metal-working fluids (Karadzic *et al.* 2006). *P. aeruginosa* has been found in non-treated bottled mineral water (Naze *et al.* 2010), tap

doi: 10.2166/wh.2011.072

water (Trautmann *et al.* 2001) and water distribution systems (Emde *et al.* 1992).

P. aeruginosa adapts to different environmental conditions. In nature, bacteria are exposed to temperature variations, and are affected by the availability of nutrients and water and the presence of toxic molecules. Reacting to these changes requires a series of rapid adaptive responses (Ramos *et al.* 2001). *P. aeruginosa* adaptation to abnormal conditions may be due to its large genome (6.3 Mb), which supports its enormous metabolic versatility and thus its adaptability to almost any challenging environment. It has been reported that *P. aeruginosa* adapts rapidly to multiple stressful environmental conditions, including starvation, desiccation and antibiotic treatments (Goh *et al.* 2002). Therefore, multiple procedures such as antibiotic susceptibility and serological and pyocin typing have been used to identify *P. aeruginosa* strains (Lautenbach *et al.* 2010). Natural environments, mainly aquatic ecosystems, usually do not completely fulfil the nutrient and energy needs of microorganisms (Blanc *et al.* 2007) leading to bacterial adaptations. Indeed, exposure of bacteria to major changes, such as temperature, salt concentration, essential nutrient supply and oxygen supply, may affect their growth conditions (Poncet *et al.* 2009). Available studies (Chau *et al.* 2002; Koning 2006) suggest the ubiquitous nature of *P. aeruginosa* as a highly versatile pathogen able to adapt to a wide range of environmental factors. With such abilities, *P. aeruginosa* strains may be capable of contaminating water supplies at several points (World Health Organization 2006; Wu & Chau 2006; Jezierska *et al.* 2011) and subsequently humans, resulting in a very serious health problem. Thus the purpose of this study was to investigate the effect of environmental conditions on *P. aeruginosa*. Specifically we wished to analyze the effect of various water microcosms on *P. aeruginosa* phenotypic response that could occur after a prolonged period of culture (12 months) under fixed (nutrients, variation of pH, temperature, etc.) factors.

MATERIALS AND METHODS

Physicochemical parameters of the water samples

This study used five different types of water sample: seawater (Sw), textile wastewater (Tww), industrial wastewater (Iww), urban wastewater (Uww) and mineral water (Mw). Water samples were prepared as previously described (Darcan *et al.* 2009). Physicochemical parameters were then determined (Chau 2002; Muttil & Chau 2007): pH, electrical conductivity (EC), sodium (Na^+), chloride (Cl^-), sulphate (SO_4^{2-}), calcium and magnesium (Ca^{2+}, Mg^{2+}), chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5). Each experiment was conducted at 25 °C, with duplicate determinations of all of the parameters.

Strain and water microcosms

The wild-type strain used in this study was *P. aeruginosa* ATCC-27853 (American Type Culture Collection). This is a laboratory prototype strain used for susceptibility controls in antibiotic resistance assays and was purchased from Charles Nicole Hospital (Tunis, Tunisia). To prepare inocula, *P. aeruginosa* ATCC-27853 cells were first inoculated into tubes containing 10 ml of Luria Bertani (LB) broth. The tubes were then incubated at 37 °C with agitation at 120 rpm until the absorbance growth reached an optical density of 1.0 at 620 nm. The bacterial cells were subsequently collected by centrifugation ($5{,}000 \times g$, 20 min), washed three times in M-9 buffer, and adjusted to a concentration of 1×10^5 CFU/ml and used to inoculate different water microcosms. Five 100 ml microcosms were prepared from Sw, Tww, Iww, Uww and Mw, which had been sterilised by filtration through a 0.22 μm pore size cellulose-ester membrane, and inoculated with 1×10^5 CFU, as previously reported (Vachée *et al.* 1997). Control microcosms were non-inoculated filtered sterile waters. All of the microcosms were incubated at 25 °C for 12 months.

Phenotypic characterisation of the stressed strains

Isolation of the colony and phenotypic tests

After 12 months, aliquots from each water microcosm were plated onto cetrimide agar and trypticasein soy agar. The plates were incubated at 37 °C for 48 h; the resulting colonies were then tested for Gram staining and oxidase reaction. Cell growth was tested using tubes containing 10 ml of nutrient broth (bio-Rad Laboratories, Marnes-la-Coquette, France), which were incubated at 37 °C and at 42 °C for 48 h. Colony morphology was visualised using Congo red agar (Römling

2001). For pyocyanin and pyoverdin pigment determination, cells were plated onto King A and King B media and incubated at 37 °C.

Biochemical and enzymatic detection

Biochemical and enzymatic reactions were performed on the isolated colonies. The identification tests for the genera and species were performed with a standard API (API 20 NE-system bioMérieux, Marcy l'Étoile, France) according to the manufacturer's instructions. Substrate assimilations were read after incubation at 37 °C for 24 and 48 h. Then, the results were interpreted by means of the identification software version 6.0 ApiWeb (bioMérieux). The strains were classified into three groups, as determined by Bosshard *et al.* (2006). The API Zym micromethod (bioMérieux) allows for the detection of 19 different enzymatic activities of the isolated strains under starvation conditions in various microcosms. The API ZYM strips were used according to the manufacturer's instructions: 65 µl samples of supernatant were added to each tube and the strips were incubated at 37 °C for 4 h, after which time API reagents Zym A and Zym B were added and the results read.

Isolation of DNA and polymerase chain reaction (PCR) amplification of the 16S–23S rDNA intergenic transcribed spacer

Isolated strains from each microcosm were cultivated on LB agar for 24 h at 30 °C, then the total nucleic acids were extracted by means of the InstaGene™ Matrix (bio-Rad Laboratories) according to the protocol described by the manufacturer. The corresponding DNA encoding 16S–23S rDNA intergenic transcribed spacer (ITS) was amplified from genomic DNA using forward primer Paer1, 5′-TCCAAA CAATCGAAAGC-3′ and reverse primer Paer2, 5′-CCGAAAACTTGCGCTTGAAC-3′. All of the amplifications were performed in a 100 PCT- thermocycler, as previously reported (Tyler *et al.* 1995). The reaction mixture (50 µl) included 1 × reaction buffer (5 µl), 0.2 mM of each deoxynucleoside triphosphate, 1 µM of forward and reverse primer, 1 µl of the DNA template, 1.5 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase. Each PCR reaction consisted of 30 amplification cycles, with an initial denaturation of 2 min at 94 °C followed successively by denaturation (30 sec) at 94 °C, annealing (30 sec) at 60 °C, and extension (1 min), ending with a 10 min extension at 72 °C and subsequent cooling to 4 °C. The molecular weight standard used was DNA Hyper Ladder II (Promega). The PCR products were loaded onto a 2% agarose gel for electrophoresis followed by ethidium bromide staining (Sambrook *et al.* 1989).

Antibiotic susceptibility

The antibiotic susceptibility test was performed by using the disk diffusion method as indicated by the Antibiogram Committee of the French Society for Microbiology (CA-SFM). Mueller-Hinton agar plates (bio-Rad Laboratories) were inoculated with a suspension of bacteria at a density of 0.5 MacFarland units (~10^6), as adjusted by a Densimat photometer (bioMérieux, Craponne, France). Antibiotic-containing disks were then placed on the Mueller-Hinton agar surface and the plates were incubated at 37 °C for 24 h. The antibiotics (bio-Rad) tested included ticarcillin (75 µg), amikacin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), gentamicin (15 µg), imipenem (10 µg), colistin (50 µg), kanamycin (30 UI) and tobramycin (10 µg). The experiments were performed with triplicate determinations of the inhibition diameter.

Pyocin typing by the spotting method

Indicator strains for pyocin typing were purchased from the Microorganisms and Bioactive Molecules Laboratory of Professor A. Boudabous, Faculty of Sciences-Tunis (Tunisia). The isolates from the water microcosms were streaked to a single colony onto trypticasein soy agar (Pronadisa, Madrid, Spain) plates and incubated overnight at 37 °C. The developed colonies were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline by measuring absorbance at 550 nm with an ultraviolet-visible light spectrophotometer (Beckman Coulter, Brea, CA, USA). A multipoint seeding system was used to dispense 1 µl volumes of the bacterial suspensions onto a set of 13 plates (diameter 90 mm), each containing 10 ml of trypticasein soy agar. Once the spots had dried, the plates were incubated at 30 °C for 6 h. Whatman filter paper disks (5 × 5 cm) were then impregnated with chloroform and the plates were placed over the discs for

15 min to allow for the evaporation of the chloroform. The plates were then exposed to open air for an additional 15 min to eliminate residual chloroform vapour. The indicator strain cultures were prepared according to Fyfe *et al.* (1984) and were placed separately onto the plates by adding a volume of 0.2 ml from each bacterial indicator to 5 ml of molten, semi-solid agar (1% peptone, 0.5% agar) held at 45 °C and poured as overlays. Once the overlays had set, the plates were incubated for 18 h at 37 °C and the pyocin types were determined by the appearance of clear inhibition zones around the bacteria. The recognition of different inhibition patterns was described by eight labelled indicators, while further subdivision into 25 possible subtypes was performed with five additional indicator strains labelled from A to E (Govan 1978). The microcosm isolates were classified as previously reported (Govan 1978). The experiment was repeated three times.

Serotyping

The strains were serotyped by agglutination testing with commercial anti-sera (bio-Rad). Serotyping is based on a slide agglutination technique that employs the first four polyvalent anti-sera and monovalent anti-sera used in the composition of polyvalent agglutination anti-sera. The 16 most common serotypes can thus be determined. Each polyvalent and monovalent antiserum was used undiluted in the slide agglutination test.

Cultures were grown on LB agar (Scharlau, Sentmenat, Spain) at 37 °C overnight and were dispersed in phosphate-buffered saline until a homogeneous suspension was obtained and slide agglutination was achieved (Raymond *et al.* 2002). The reaction was read when the mixture exhibited clear aggregation and settling occurred. In contrast, the control cells remained uniformly suspended in the solution. The experiment was repeated three times.

RESULTS AND DISCUSSION

Morphological responses of *P. aeruginosa*

Table 1 presents the physicochemical parameters of the water samples used to prepare microcosms with a pH ranging between 7.5 and 8.5. The Sw sample displayed high levels of electrical conductivity, salinity and ionic concentrations compared to the other water samples. This ionic composition of the seawater may exert osmotic pressure on the *P. aeruginosa* growth. The lowest values in terms of physicochemical parameters were shown by Mw (Table 1). Relatively high values of Cl^--ions and SO_4^{2-}-ions were found in Iww and Tww samples, respectively.

Table 1 | Physicochemical parameters of water samples

	Water samples				
Parameters	**Sw**	**Tww**	**Iww**	**Uww**	**Mw**
pH	8.1	8.5	7.6	7.7	7.5
EC μS/cm	50	7.8	1.5	1.8	1.3
Cl^- mg/l	20,519	603.5	887.5	426	23
SO_4^{2-} mg/l	4,416	1,430	312	403	22
Na^+ mg/l	16,100	2,823	101.4	262.2	235
$(Ca^{2+} + Mg^{2+})$ mg/l	2,220	180	210	225	75
COD mg/l	3,430	182	115	43	0
BOD_5 mg/l	10	35	50	20	0

Sw, seawater; Tww, textile wastewater; Iww, industrial wastewater; Uww, urban wastewater; Mw, mineral water; EC, electric conductivity; SO_4^{2-}, sulphate; Cl^-, chloride ion; Na^+, sodium ion; $(Ca^{2+} + Mg^{2+})$, calcium and magnesium; COD, chemical oxygen demand; BOD_5, biological oxygen demand over five days. The experiment was performed at 25 °C. Values given for all parameters are average of duplicate determinations.

The different water microcosms were inoculated with *P. aeruginosa* ATCC-27853 and incubated for 12 months, then the stressed cells isolated from the microcosms were seeded on cetrimide agar medium. When grown on Congo red agar, ATCC-27853 (wild type) showed dry, dark red colonies (Figure 1(a)), while the stressed strain isolated from seawater generated round, red, smooth and slightly opaque colonies (Figure 1(b)). The colony form of the stressed strains isolated from the Tww, Iww, Uww and Mw microcosms remained identical to that of the wild-type colonies. As shown by Solano *et al.* (2002), red colonies are the result of Congo red adsorption.

The stressed cells isolated from all of the water microcosms maintained a Gram-negative stain, were oxidase-positive, catalase-positive and were able to grow in nutrient broth at 42 °C, producing pyocyanin and pyoverdin pigment. Interestingly, the isolate from the Sw microcosm was unable to produce pyocyanin pigment on King A medium. Moreover, the organism remained cultivable following

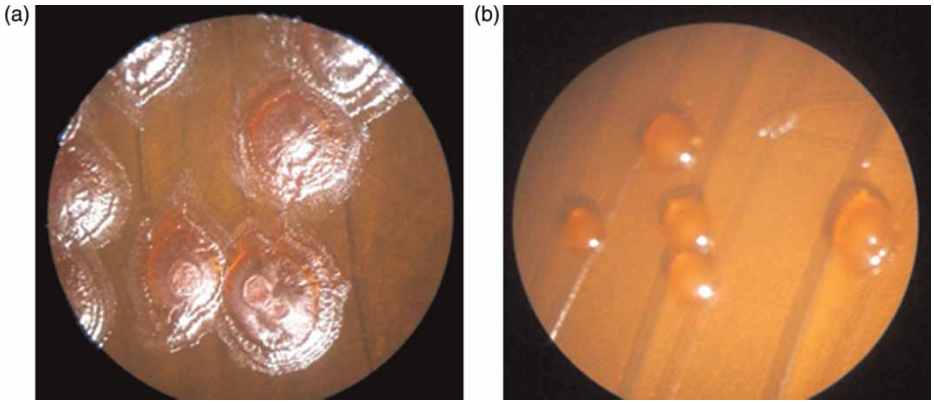

Figure 1 | Colony morphology of *P. aeruginosa* ATCC-27853. (a) wild-type and (b) stressed isolate from seawater microcosms and grown on Congo red agar at 37 °C.

starvation in a water microcosm characterised by a nutrient deficit, a significant level of salinity and the presence of polluting substances. This demonstrates the capacity of wild-type *P. aeruginosa* to adapt to the different physicochemical parameters of water. It confirms the ubiquitous nature of *P. aeruginosa* (Green *et al.* 1974).

Biochemical and enzymatic responses

Starvation of the *P. aeruginosa* ATCC-27853 in the Sw, Tww, Iww, Uww and Mw microcosms enhanced the production of urease and the hydrolysis of aesculin substrate by producing beta-glucosidase enzymes. The strain isolated from the Mw microcosm became urease positive and aesculin hydrolysis positive through the production of beta-glucosidase and with the assimilation of mannose as a carbon source.

According to the biochemical analyses (Table 2), the *P. aeruginosa* ATCC-27853 (Si) strain, was identified at a rate of 99.9% and a *T* value of 0.9. The stressed organisms isolated from Sw, Tww, Iww and Uww after 12 months demonstrated identical biochemical profiles with a very good identification rate of 99.9% and a *T* value 0.58. In contrast, the isolate from the Mw microcosm was identified at 73.8% with a *T* value of 0.25; this result shows an uncertain biochemical profile, although the genus was determined as *Pseudomonas*. This suggests a possible stressful effect of the Mw microcosm on *P. aeruginosa* as compared with the other microcosms. Such an effect could be due to mineral water's nutrient-free quality. However, further studies are necessary to determine those factors in the Mw microcosm leading to modifications of the *P. aeruginosa* biochemical profile (Morais *et al.* 1997).

Although the API 20NE system was useful in identifying bacteria under the imposed stress conditions, there were some limitations. As shown in this study, the urease reaction was a variable factor in *P. aeruginosa*

Table 2 | Pyocin typing and subtyping of *P. aeruginosa* ATCC-27853 before and after culture in different water microcosms against indicator strains

	1	2	3	4	5	6	7	8	Types	A	B	C	D	E	Subtypes
Si	–	–	+	+	+	–	+	+	24	+	+	+	–	+	n
S1	–	–	(–)	+	+	–	+	+	(83)	+	+	+	(+)	+	(a)
S2	–	–	+	+	+	–	+	+	24	+	+	+	–	+	n
S3	–	–	+	+	+	–	+	+	24	+	+	+	–	+	n
S4	–	–	+	+	+	–	+	+	24	+	+	+	–	+	n
S5	–	–	+	+	+	–	+	+	24	+	+	+	–	+	n

Typing was investigated using API 20 NE-synstem. (), modified character; Si, characteristic of *P. aeruginosa* ATCC-27853 (wild type); S1, S2, S3, S4 and S5 inhibition patterns of starved *P. aeruginosa*, respectively, isolated from sea water, textile wastewater, industrial wastewater, urban wastewater and mineral water microcosms; +, inhibition of indicator strain; –, no inhibition; 1, 2, 3, 4, 5, 6, 7 and 8, strains indicator pyocin types; A, B, C, D and E, strains indicator pyocin subtypes. The experiment was realised in triplicate determinations.

ATCC-27853 after starvation in the water microcosms. This is in agreement with previous reports (Bakhrouf *et al.* 1989) showing that *P. aeruginosa* acquired new activities, such as urease synthesis for the hydrolysis of urea, aesculin hydrolase and mannose assimilation. In addition, Dominguez-Bello *et al.* (2000) found that 72% of *P. aeruginosa* isolates of clinical origin can be urease positive. Data of the present study and those available in the literature suggest that biochemical profile of *P. aeruginosa* could be modulated through pressure exerted by the environment on cell activity. It has been shown that bacteria routinely alter their physiological state to tolerate or exploit local environments in order to compete and survive in nature (Koning 2006; Lucchetti-Miganeh *et al.* 2008). However, all of the stressed organisms isolated from the various water microcosms do maintain stable activities, such as glucose oxidation and the production of gelatin hydrolase, nitrate reductase, arginine dihydrolase and lipase.

The prototype strain ATCC-27853 showed activity by five enzymes. After a starvation period in various water media, the isolates displayed no changes in the following enzymatic activities: alkaline phosphatase, esterase, esterase lipase, lipase and leucine arylamidase, as tested by API ZYM. These results support the capability of *P. aeruginosa* ATCC-27853 to not only survive but to retain most of the metabolic activities in the different water samples, except for the seawater environment, which effected more phenotypic variation in the organisms. The stability of the enzymatic functions may be explained by the fact that mutations did not affect the genes of the wild-type strain. In fact, extracellular lipase production was also shown to be positively controlled by RsmA in *P. aeruginosa* (Heurlier *et al.* 2004). Moreover, marine *P. aeruginosa* living at low nutrient levels, high salinity and alkaline pH (~8) conditions were different from those isolated from fresh, clinical and coastal waters as showed by Khan *et al.* (2008). However, for the fittest variants to be selected, they must survive over a sufficient period of time under the new conditions. Indeed, Izrael-Zivkovic *et al.* (2009) reported that even though ATCC-27853 was not isolated from extreme conditions, it did display extracellular lipase with optimal characteristics, namely, temperature 50 °C and pH 9.3 in a water solution.

Molecular detection of *P. aeruginosa*

Starvation in water microcosms affected the morphological, biochemical, antibiotic, pyocin and serotype changes in the wild-type strain. It was, however, deemed necessary to carry out molecular identification in order to confirm the microbial species. PCR amplification of the 16S–23S rDNA ITS region with primers Paer1 and Paer2 generated a 181 bp DNA fragment from all of the strains isolated from the water microcosms and were identified as *P. aeruginosa* (Figure 2). Molecular identification by 16S–23S rDNA ITS was therefore very important in our determination of the species of isolate isolated from the aquatic environments. In terms of adaptation to the nutrient deficiency, it appears that the strain adopted new metabolic strategies during its stay in the different water samples. However, these metabolic strategies are still to be identified. Isolates can be genetically identical yet phenotypically different by producing various lipids, proteins and carbohydrates (Poncet *et al.* 2009). It was thus important that molecular detection be performed first for the bacteria isolated from the water and particularly the seawater environment.

Antibiotic susceptibility

The wild-type *P. aeruginosa* strain displayed natural resistance to ticarcillin, ceftazidime and imipenem. In addition,

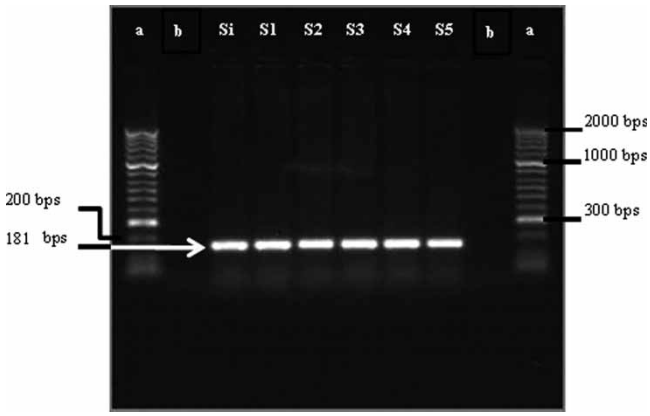

Figure 2 | Amplification (PCR) of 16S–23S intergenic sequence in stressed *P. aeruginosa*. Agarose gel electrophoresis showing a band representing the amplified DNA fragment of an ITS gene. (a) molecular weight marker; (b) control; (bps) base pairs; (Si) *P. aeruginosa* ATCC-27853 (wild type); S1, S2, S3, and S4 bands of each stressed isolate from seawater, textile wastewater, industrial wastewater, urban wastewater and mineral water microcosms, respectively.

Fass *et al*. (1996) confirmed the sensitivity of *P. aeruginosa* to tobramycin. All of the isolates from the water microcosms maintained resistance to gentamicin, kanamycin and cefotaxime and were sensitive to ciprofloxacin and amikacin, which are related to antibiotic groups quinolones and aminosides (Table 3). The starvation of ATCC-27853 in various water media did, however, enhance resistance against beta-lactam antibiotics (Chen *et al*. 1995). This resistance may be due to the loss or the closing of porine D2. Livermore (2001) reported that a loss of OprD and an upregulation of MexA–MexB–OprM may occur in any *P. aeruginosa* strain through simple mutation. The isolate from the seawater microcosm was found to be resistant to imipenem, ticarcillin, ceftazidime, tobramycin and colistin. This phenotype resistance may also be explained by the high level of sulphate and sodium ions. Thus the seawater environment promoted *P. aeruginosa* adaptation/resistance to antibiotics. The isolates from the Sw and Tww samples became resistant to tobramycin. Indeed, according to Hocquet *et al*. (2003), an adaptive resistance to aminosides implies an active MexX–MexY–OprM efflux system as well as another efflux channel of the external membrane, called Opm6, which ensures an increased resistance to aminosides. In addition, Magnet *et al*. (2003) showed that 6′-*N*-acetyltransferase contributes to the resistance to aminosides, and particularly tobramycin by antibiotic neutralisation. On the other hand, Li *et al*. (2000) showed that the majority of *P. aeruginosa* strains acquired resistance to tetracycline by both active efflux and low membrane permeability. A clear interpretation of the antibiotic susceptibility tests is therefore crucial to better appreciate the effect of antimicrobial agents on bacteria such as *P. aeruginosa*. Increasing resistance of starved ATCC-27853 to antibiotics may be attributed to the actions of several regulating genes (Kümmerer 2004).

Table 3 | Antibiotic effect on the stressed *P. aeruginosa* ATCC-27853

	Antibiotic									
	IPM	TIC	CTX	CAZ	AN	TM	GM	K	CIP	CL
Si	S	S	R	S	S	S	R	R	S	S
S1	(R)	(R)	R	(R)	S	(R)	R	R	S	(R)
S2	(R)	(R)	R	(R)	S	(R)	R	R	S	S
S3	(R)	(R)	R	(R)	S	S	R	R	S	S
S4	(R)	S	R	S	S	S	R	R	S	S
S5	(R)	(R)	R	(R)	S	S	R	R	S	S

( ), modified character; Si, antibiotic susceptibility of *P. aeruginosa* ATCC-27853 (wild type); S1, S2, S3, S4 and S5 antibiotic susceptibility of starved *P. aeruginosa*, respectively, isolated from sea water, textile wastewater, industrial wastewater, urban wastewater and mineral water microcosms; S, sensitive; R, resistant; IPM, imipinem; TIC, ticarcillin; CTX, cefotaxime; CAZ, ceftazidime; AN, amikacin; TM, tobramycin; GM, gentamicin; K, kanamycin; CIP, ciprofloxacin; CL, colistin. The experiment was performed with triplicate determinations of inhibition diameter and the average of measures was taken.

Pyocin production and serotyping

The results (Table 2) show a change of pyocin type and subtype of *P. aeruginosa* ATCC-27853 from 24/n to 83/a following starvation in the Sw microcosm. In contrast, the stressed isolates from the Tww, Iww and Mw microcosms preserved the same pyocin (24/n). In this study, pyocin production was influenced by the nature of the environment and particularly by the Sw environment. This production is regulated by a *prtN* activator gene that activates the expression of several pyocin genes and by a *prtR* repressor gene that inhibits the form of the *prtN* gene (Matsui *et al*. 1993).

An agglutination serotype assay was performed with polyvalent anti-sera which included monovalent anti-sera O1 through O6. The serogroup of the stressed strain isolated from the Sw microcosm altered from O6 to O1, yet remained unchanged (O1) in the other water microcosms (Table 4). The change of serotype may be a result of mutations due to the O-antigen change (Spencer *et al*. 2003). Thus seawater was the more stressing biotope for *P. aeruginosa* ATCC-27853, as its survival was followed by

Table 4 | Serotype group of *P. aeruginosa* ATCC-27853 before and after culture in different water microcosms

		PMA				
	Autoagglutination	P1	P3	P4	P6	Serotype
Si	–	–	–	–	+	O6
S1	–	(+)	–	–	(–)	(O1)
S2	–	–	–	–	+	O6
S3	–	–	–	–	+	O6
S4	–	–	–	–	+	O6
S5	–	–	–	–	+	O6

( ), modified character; Si, serotype of *P. aeruginosa* ATCC-27853 (wild type); S1, S2, S3, S4 and S5 serotypes of stressed *P. aeruginosa*, respectively, isolated from sea water, textile wastewater, industrial wastewater, urban wastewater and mineral water microcosms; +, agglutination; –, no agglutination, PMA, serums mixtures; P1, P3, P4 and P6, monovalent sera. The experiment was performed in triplicate.

an altered lipopolysaccharide composition that involved a change of the serotype.

Although serotyping is a common method of phenotyping, its value in relation to the epidemiology may be limited. Indeed Rahim *et al*. (2000) showed that the frequency of serotype changes *in vivo*, is probably due to environmental influences on survival that cause deterioration of the structure of the O antigen of lipopolysaccharide. Seroconversion of a serotype is possible; however, the seroconversion of serotype O6 to O1 was not addressed in our study. It may be explained as described by Rahim *et al*. (2000).

CONCLUSIONS

Our study shows that starvation of wild-type *P. aeruginosa* in various water microcosms gives different isolates with varied phenotypic characters related to the culture environment. We demonstrated that growth of *P. aeruginosa* in various water microcosms leads to bacteria adapting to their environment through biochemical profile change, morphological variation, antibiotic resistance, type of pyocin and serotype modulations in relation to the aquatic environments. This study clearly demonstrated that environmental conditions promote microbial phenotypic changes that may lead to drug resistance of microorganisms such as *P. aeruginosa*. However, further molecular studies could be performed to explain how phenotypic changes and resistance acquisition occur in relation to the environmental conditions.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Society of Exploitation and Distribution of Water in Tunisia; and the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Program (MR).

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First received 3 May 2011; accepted in revised form 23 September 2011. Available online 20 December 2012