Research Paper

A comparative study of coastal and clinical isolates of *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium having a versatile metabolic potential and great ecological and clinical significance. The geographical distribution of *P. aeruginosa* has revealed the existence of an unbiased genetic arrangement in terrestrial isolates. In contrast, there are very few reports about *P. aeruginosa* strains from marine environments. The present work was aimed at studying the distribution of *P. aeruginosa* in coastal waters along the Indian Peninsula and understanding the environmental influence on genotypic, metabolic and phenotypic characteristics by comparing marine and clinical isolates. Of the 785 marine isolates obtained on selective media, only 32 (~4.1%) were identified as *P. aeruginosa*, based on their fatty acid methyl ester profiles. A low Euclidian distance value (< 2.5) obtained from chemotaxonomic analysis suggested that all the environmental (coastal and marine) isolates originated from a single species. While UPGMA analyses of AP-PCR and phenotypic profiles separated the environmental and clinical isolates, fatty acid biotyping showed overlapping between most clinical and environmental isolates. Our study revealed the genetic diversity among different environmental isolates of *P. aeruginosa.* While biogeographical separation was not evident based solely on phenotypic and metabolic typing, genomic and metatranscriptomic studies are more likely to show differences between these isolates. Thus, newer and more insightful methods are required to understand the ecological distribution of this complex group of bacteria.

Key words: *Pseudomonas aeruginosa*, biogeography, FAME profiling, arbitrarily primed PCR, environmental distribution.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative γ-proteobacterium having a large and flexible genome (> 6.2 Mb) with a high number of protein-coding genes (5,570 ORFs) matching simpler eukaryotes (Stover *et al.*, 2000). Its influential adaptive capacity and metabolic potential allow this bacterium to live in very dissimilar environments (Römling *et al.*, 1994; Finnan *et al.*, 2004; LaBaer *et al.*, 2004; Pirnay *et al.*, 2005; Shen *et al.*, 2006; Khan *et al.*, 2008). The distribution of this species suggests a net-like population structure with equivalent genotypes and functionally similar characters elicited by both clinical and environmental strains without any specialisation (Römling *et al.*, 1994; Alonso *et al.*, 1999; Kiewitz and Tummler, 2000; Pirnay *et al.*, 2005). The species' indigenous adaptation in marine environments was a matter of debate until its presence was first reported by Kimata *et al.* (2004). Recent isolation of *P. aeruginosa* strains from an open-ocean site has shown that they possess a unique genotype. Thus, the geographical origin of the strains is reflected in their phylogeny (Khan *et al.*, 2007; Khan *et al.*, 2008; Nonaka *et al.*, 2010).

Although *P. aeruginosa* is an extensively studied organism, the bacterium in its natural reservoirs has a lot to

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explore from ecological and medical perspectives (Nonaka *et al.*, 2010). Considering Indian region, the information on the occurrence and genotypic or phenotypic characterisation of environmental *P. aeruginosa* isolates is very limited, except for a few reports from the east coasts of India (Nallathambi *et al.*, 2002; Mohandass and LokaBharathi, 2003; Sivaraj *et al.*, 2012). Environmental strains of *P. aeruginosa* are presumably less pathogenic, and therefore handling of them may be less risky compared to clinical strains. However, site-specific contamination, *e.g.*, through sewage or domestic waste disposal, may introduce clinical strains in the environment. A better understanding of the distribution of *P. aeruginosa* and the identification of characteristics enabling differentiation between environmental and clinical strains without the need for complex methods will allow easier strain development for industrial and medical use. Since *P. aeruginosa* demonstrates a net-like population structure, we hypothesise that environmental strains collected from similar niches might exhibit nearly identical phenotypes, but, are likely to show differences from clinical strains at the genetic level. Hence, there is a need for further investigation on the biogeographical distribution of *P. aeruginosa* isolated from diverse geographical locations.

This study aims to understand the prevalence of *P. aeruginosa* in Indian coastal waters and to further investigate the influence of the environment on genetic, metabolic and phenotypic characteristics of marine isolates in comparison with clinical isolates of the species. To characterise the isolates, we used conventional phenotypic assays (antibiotic susceptibility, pyocyanin and exoenzyme production), along with arbitrarily primed PCR (AP-PCR) and fatty acid methyl ester (FAME) analyses which provide greater specificity and better resolution for distinguishing bacterial isolates (Mahenthiralingam *et al.*, 1996; Khan *et al.*, 2007; Chao *et al.*, 2010). Since *P. aeruginosa* is well-known for multidrug resistance, analysis of antibiotic susceptibility patterns and pathogenic characters such as exoenzymes (haemolysin and protease) and pyocyanin production would yield an insight into their distribution and more likely to reflect such a potential in environmental isolates (Alonso *et al.*, 1999; Stehling *et al.*, 2010; Nonaka *et al.*, 2010).

Materials and Methods

Sample collection

Sub-surface seawater samples were collected in sterile containers from four different sites located in Indian coastal regions, Mumbai (M), Cochin (C), Kakinada (K) (referred to as coastal) and Andaman (A) (referred to as marine) (Figure 1). Hydrographic parameters such as salinity, pH and temperature of the water were recorded at each site (Table 1).
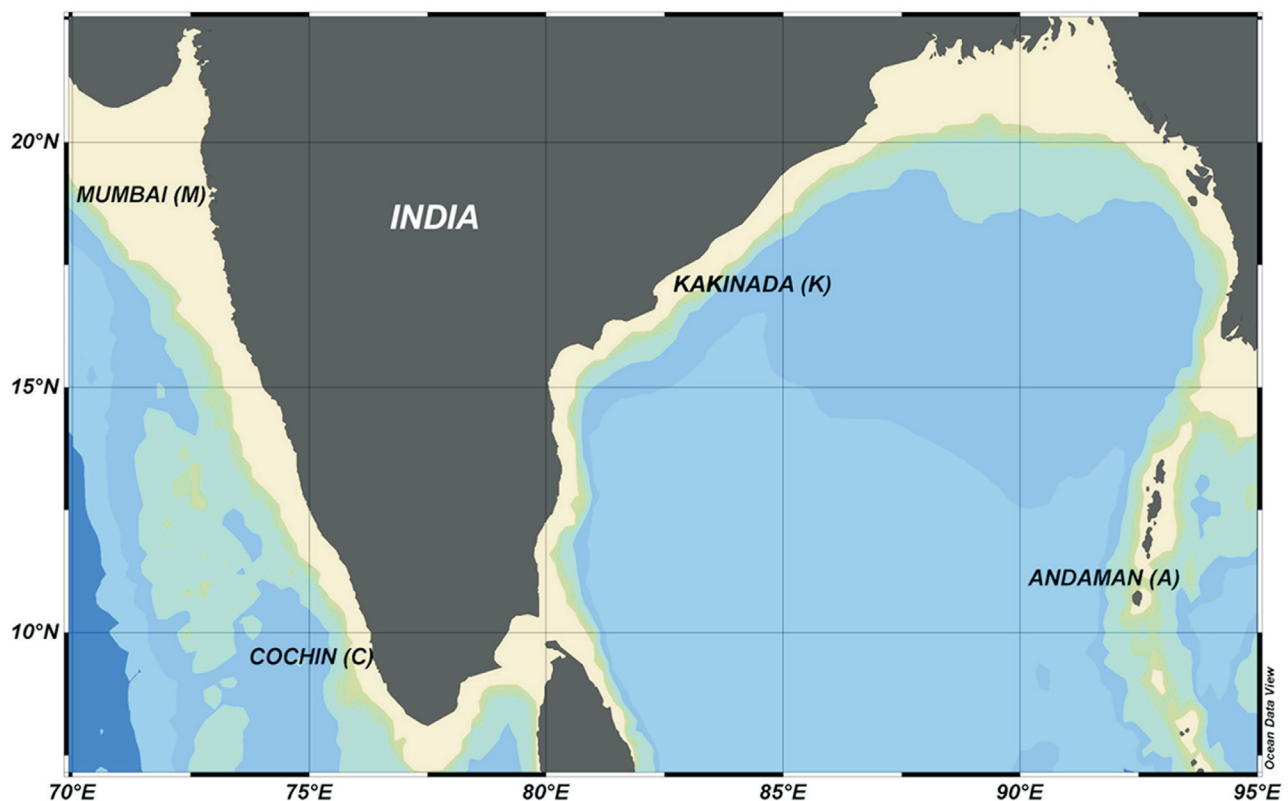
Figure 1 - Locations of the sample collection sites along the Indian coast.

Table 1 - Location, source, number and isolate name of *Pseudomonas aeruginosa*.

Origin	Location	Source	Hydrographic characteristics			Total number of isolates	Number of isolates at particular station	Isolate identity
			pH	Salinity (‰)	Temperature (°C)			
Marine	Andaman	Water	8	35	28	7	7	A1 - A7
Coastal	Mumbai	Water	8.4	36	26	25	8	M1 - M8
	Kakinada	Water	7.7	34	31		10	K1 - K10
	Cochin	Water	7.8	35	30		7	C1 - C7
Clinical	Hospital	Urine	NA	NA	NA	7	2	CLN2 & CLN5
	NA	Pus	NA	NA	NA		5	CLN1, CLN3, CLN4, CLN6 & CLN7
Reference	NA	Outer ear infection	NA	NA	NA	1	1	MTCC 1688

NA – Not Applicable.

Mumbai (M) is a coastal station on the central west coast of India categorised as a "hot spot" by the Government of India's surveillance program "Coastal ocean monitoring and prediction system" (COMAPS). This region receives industrial wastes and effluent from refineries and petrochemical plants, loading and unloading of crude oil and its products in the Mumbai port, and the indiscriminate release of treated and untreated sewages. Cochin (C) is a coastal station located along the southwest coast of India and characterised by anthropogenic inputs to the estuarine ecosystem of industrial and domestic wastes including heavy metals. It comes under a moderately polluted site based on the COMAPS report. Kakinada (K) Bay is located on the east coast of the Indian Peninsula and is subjected to anthropogenic inputs from agricultural processes, industrial development and port expansion. Recently, it has been categorised as a polluted environment but the level of pollution is very negligible compared to the Mumbai and Cochin stations. Andaman (A) is a pristine marine station on the Andaman Sea. This offshore station has been classified as a pollution-free zone.

Isolation and identification

Sub-surface seawater samples (5 L) were collected from each location and filtered through 0.2 µm polycarbonate filters. The filters were incubated at 28 °C for 48 to 72 h on nalidixic acid cetrimide (NAC) agar (Himedia, India) plates. All media used in this study were prepared with 50% seawater. Colonies showing unique pigmentation (pyocyanin) and other characteristics typical of *P. aeruginosa* were picked, inoculated in cetrimide kanamycin nalidixic acid (CKNA) agar (Himedia, India) plates and incubated at 42 °C for 48 h as recommended by Kimata *et al.* (2004). The environmental isolates were identified using standard biochemical tests (Holt *et al.*, 1994) and the Sherlock Microbial Identification System (MIS) (MIDI Inc., USA). Additionally, a reference strain (MTCC 1688) and 7 clinical isolates (obtained from the PVS Hospital, Ernakulam, India) used in the study were confirmed by the same methods. All isolates were preserved as 20% glycerol stocks in nutrient broth at -80 °C throughout the study. All experiments were done in triplicates unless indicated otherwise.

Fatty acid methyl ester (FAME) typing

FAME typing was carried out on 24 h old cultures grown at 28 °C on soybean casein digest agar (Himedia, India) plates. Approximately 40 mg of bacterial biomass was harvested from the culture plates to extract fatty acids. Methyl esters were prepared as described by Sasser (2006), and profiling and identification were carried out on an Agilent 6890N (Agilent Technologies) gas chromatograph equipped with a flame ionization detector, an autosampler and an injector. Relative peak areas were determined by using a standard program. The FAME profiles were com-

pared, and a dendrogram was constructed by the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) using the Sherlock MIS software. More than 85% of the total peak area was named, and the standard FAME nomenclature was used based on the structural properties as described by Duran *et al.* (2006). The relatedness between the strains was expressed as the Euclidian distance (ED), the distance in multi-dimensional space between the fatty acid compositions of the strains compared.

Arbitrarily primed PCR (AP-PCR) genotyping

DNA was extracted from 5 mL of overnight cultures of all *P. aeruginosa* isolates grown in LB medium by using the CTAB/NaCl method (Ausubel *et al.*, 2002), and the DNA extracted was quantified using a BioPhotometer (Eppendorf, Germany). AP-PCR was performed using primer 208 (5'-ACGGCCGACC-3') which was selected from the 8 primers described by Mahenthiralingam *et al.* (1996) based on its ability to provide good reproduction and maximum differentiation between the strains (Supplementary Table 1). Each 25 µL PCR reaction contained 35 ng of template DNA, 40 pmol oligonucleotide (Bioserve Biotechnologies, India), 1.5 U of *Taq* polymerase, 250 µM dNTPs, and 2.5 µL of 10X reaction buffer (Genei, India). Amplification was carried out in two stages in an Eppendorf thermal cycler (Germany): (1) 4 cycles of denaturation at 94 °C for 5 min, annealing at 36 °C for 5 min and elongation at 72 °C for 5 min, followed by (2) 30 cycles at 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel, then stained with ethidium bromide and documented using a Kodak Gel Logic 1500 imaging system (Eastman Kodak Company, USA). The bands were coded based on their sizes (range 0.2-2 kb) in order to minimise errors. The results of amplification were recorded as positive or negative. The similarities among the strains were estimated by the Dice coefficient (negative matches excluded), and the strains were grouped by UPGMA to facilitate the plotting of a dendrogram.

Phenotypic characterisation

Antibiotic susceptibility

The susceptibility patterns of all isolates were tested using discs (Hicomb, Himedia, India) containing 13 antibiotics belonging to 8 different groups, including penicillin, glycopeptide, fluoroquinolone, cyclic polypeptide, tetracycline, cephalosporin, aminoglycoside, and sulfamethoxazole. Minimum inhibitory concentrations (MIC) for the antibiotics were determined on Mueller-Hinton agar (Himedia, India) using the disc diffusion method (Bauer *et al.*, 1966). MIC (µg/mL) of a particular antibiotic was read from the intersection of the ellipse-formed zone of inhibition with the value marked.

Pyocyanin assay

The pyocyanin assay was carried out in glycerol-alanine medium (per litre: 10 g DL-alanine, 20 mL glycerol, 0.139 g K_2HPO_4, 4.06 g $MgCl_2.6H_2O$, 14.2 g Na_2SO_4, 0.1 g ferric citrate, pH 7.0 ± 0.2) inoculated with 1% of overnight grown cultures. The suspensions were incubated on a rotary shaker at room temperature for 24 h. After incubation, the cells were centrifuged at 8,000 rpm for 10 min. An equal volume of chloroform was added to 5 mL of cell-free supernatant. The lower organic layer was collected and acidified with 1.5 mL of 0.2 N HCl to yield red colour. Absorbance was measured at A_{520}, and the concentration was determined using a multiplication factor of 17.072 as calculated previously by Essar *et al.* (1990) and expressed in µg/mL of pyocyanin.

Screening of proteolytic and haemolytic activities

To determine the proteolytic and haemolytic activities, 5 µL of overnight cultures grown in Luria-Bertani broth (Himedia, India) were spotted on casein milk agar and 5% blood agar plates, respectively (Alonso *et al.*, 1999). After 48 h of incubation at room temperature, activities were measured as the diameters of clear halos formed around bacterial colonies.

The isolates were grouped using UPGMA cluster analysis, and their phenotypic similarities were calculated with the Bray-Curtis similarity coefficient using the PRIMER-5 software (Clarke and Gorley, 2001). Statistical evaluation was carried out with the SPSS 20.0 software (SPSS Inc, Chicago, USA). Analyses were performed in triplicate, and the means of all parameters were examined for significance (p < 0.05) by analysis of variance (ANOVA).

Results

Isolation and identification

Pseudomonas aeruginosa strains were isolated from sub-surface seawater samples collected from four stations located in India. The number of *P. aeruginosa* cells detected in the coastal waters was low and ranged from a non-detectable level to 5 cfu/L of water. Of the 785 initial colonies isolated on NAC agar, 264 colonies with unique pigmentation similar to that of *P. aeruginosa* were selected and streaked on fresh CKNA agar plates. Among these, only 32 isolates showed characteristics typical of *P. aeruginosa*, *i.e.*, flat greenish colonies (2-4 mm in diameter) with irregular edges and typical metallic lustre on nutrient agar and also growing on CKNA agar at 42 °C. The isolates were further confirmed by biochemical tests and using the Sherlock MIS (Table 1). All isolates were Gram-negative rods with positive test reactions for oxidase, catalase, nitrate reductase and citrate production and negative in the methyl red, indole and Voges-Proskauer tests.

FAME profiling

A total of 16 to 20 different fatty acids were detected in the isolates (Supplementary Table 2). Of the 12 signature fatty acids of *P. aeruginosa,* 11 fatty acids [$C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ ω7c, $C_{12:0\ 2OH}$, $C_{12:0\ 3OH}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c, and Summed Feature 3 (SF3)] were found in all 32 isolates, except $C_{10:0\ 3OH}$ which was not detected in 4 Mumbai isolates (M1, M2, M3, and M8). In addition to the signature fatty acids, two more fatty acids ($C_{17:0}$ and $C_{17:1}$ ω8c) were detected in all isolates. $C_{18:1}$ ω7c (vaccenic acid) was the major fatty acid present in all isolates and contributing up to 43% of the total cellular fatty acid composition, followed by $C_{16:0}$ (palmitic acid) with 26.73% (Table 2). The proportion of monounsaturated, saturated, hydroxy, and cyclopropane fatty acids detected in the isolates was 42.29%, 30.88%, 11.16% and 1.39%, respectively. In addition to these fatty acids, a branched fatty acid ($C_{17:0}$ iso, isomargaric acid) was also detected in the reference strain MTCC 1688 and two isolates from Kakinada, K10 and K7.

Euclidian distances between all *P. aeruginosa* isolates were below 10, indicating that all belonged to the same species (Figure 2). Isolate C5 from Cochin was the only isolate that showed an ED value > 7.5. The rest of isolates with ED values of 2.5 were grouped into 8 different clusters, and the majority showed similarities in their fatty acid profiles, regardless of their geographical origin.

Genotyping

A total of 18 different DNA fragments (sizes between 0.2 and 2 kb) were detected by AP-PCR. UPGMA analysis grouped the isolates into 9 clusters at the 50% similarity level (Figure 3). Although the environmental isolates fell in different clusters, isolates from the same sites frequently occurred in the same clusters. For instance, clusters R1 and R3 mostly contained Mumbai and Cochin isolates and clusters R4 and R5 mostly included Andaman and Kakinada isolates, respectively. The maximum heterogeneity was observed for the isolates from Mumbai and Cochin. However, site specificity was observed at higher similarity levels, which resulted in geographically separated clusters for each site.

Table 2 - Details of total cellular fatty acid composition (%) of the analysed isolates.

Locations	Clinical isolates	Andaman	Kakinada	Mumbai	Cochin	Reference strain	Average
Unknown 11.799	0	0.01	0.04	0.02	0.01	0	0.01
$C_{11:0\ 3OH}$	0.02	0.01	0.03	0.03	0	0	0.02
SF2	0.05	0.01	0.03	0.06	0	0	0.03
$C_{16:1\ \omega 5c}$	0.02	0.03	0.02	0.02	0	0.13	0.04
$C_{18:1}$ ω9c	0.07	0.06	0.03	0.13	0	0.16	0.08
Unknown 12.484	0.14	0.06	0.16	0.18	0.14	0	0.11
$C_{10:0}$	0.17	0.23	0.21	0.12	0.22	0	0.16
$C_{17:0}$	0.29	0.14	0.18	0.18	0.2	0.15	0.19
$C_{17:1}$ ω8c	0.38	0.18	0.23	0.19	0.21	0.27	0.24
$C_{15:0}$	0.34	0.28	0.36	0.32	0.37	0.45	0.35
$C_{12:1\ 3OH}$	0.54	0.35	0.55	0.58	0.39	0	0.40
$C_{17:0\ CYCLO}$	0.52	0.65	0.48	0.52	0.74	0.59	0.58
$C_{14:0}$	0.64	0.63	0.55	0.56	0.62	0.86	0.64
$C_{18:0}$	0.81	0.68	0.79	0.82	0.79	0.63	0.75
$C_{19:0\ CYCLO}$ ω8c	0.7	0.86	0.6	0.58	1.15	0.99	0.81
$C_{10:0\ 3OH}$	2.86	2.72	3	1.52	2.56	2.43	2.52
$C_{12:0}$	2.77	2.8	2.95	2.78	2.87	3.2	2.90
$C_{12:0\ 3OH}$	3.91	3.84	4.04	4.13	3.97	3.84	3.96
$C_{12:0\ 2OH}$	4.44	4.07	4.22	4.28	4.24	4.31	4.26
SF3	13.66	14.17	12.99	13.7	13.78	16.5	14.13
$C_{16:0}$	26.52	25.33	26.73	26.28	26.32	24.16	25.89
$C_{18:1}$ ω7c	41.15	42.89	41.81	43	41.42	41.33	41.93

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the MIDI system.

Summed Feature 2 (SF2) comprised of $C_{16:1\ ISO\ I}$ and/or $C_{14:0\ 3OH}$.

Summed Feature 3 (SF3) comprised of $C_{15:0\ ISO\ 2OH}$ and/or $C_{16:1}$ ω7c.
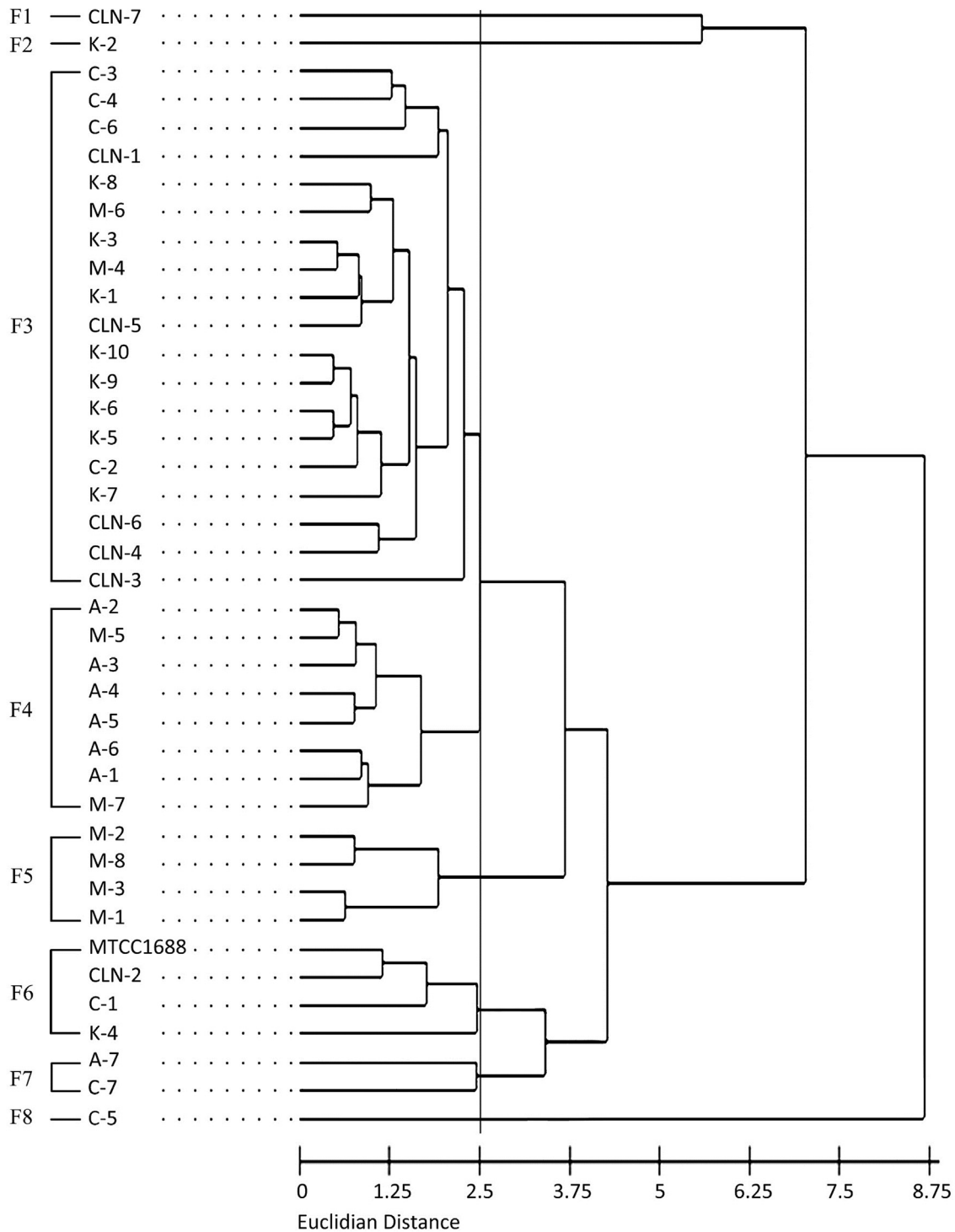
Figure 2 - Dendrogram based on the fatty acid profiles of the *P. aeruginosa* isolates. M, Mumbai; C, Cochin; K, Kakinada; A, Andaman; CLN, clinical isolates. F1-F8 indicate the clusters formed by the isolates with the threshold ED value of 2.5, based on their FAME profiles.

Phenotypic characterisation

Susceptibility patterns of the *P. aeruginosa* strains varied depending on the family of antibiotics. While all isolates were highly susceptible to the quinolone, polymyxin and aminoglycoside groups of antibiotics (0.15-

15.83 µg/mL), they were moderately susceptible to penicillin antibiotics (10.28-43.5 µg/mL). Low susceptibility/high resistance (36.52 - 135 µg/mL) was observed for the cephalosporin (Cefepime) and tetracycline families. Most environmental isolates exhibited similar or higher resistances to

Dice (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
RAPD-208
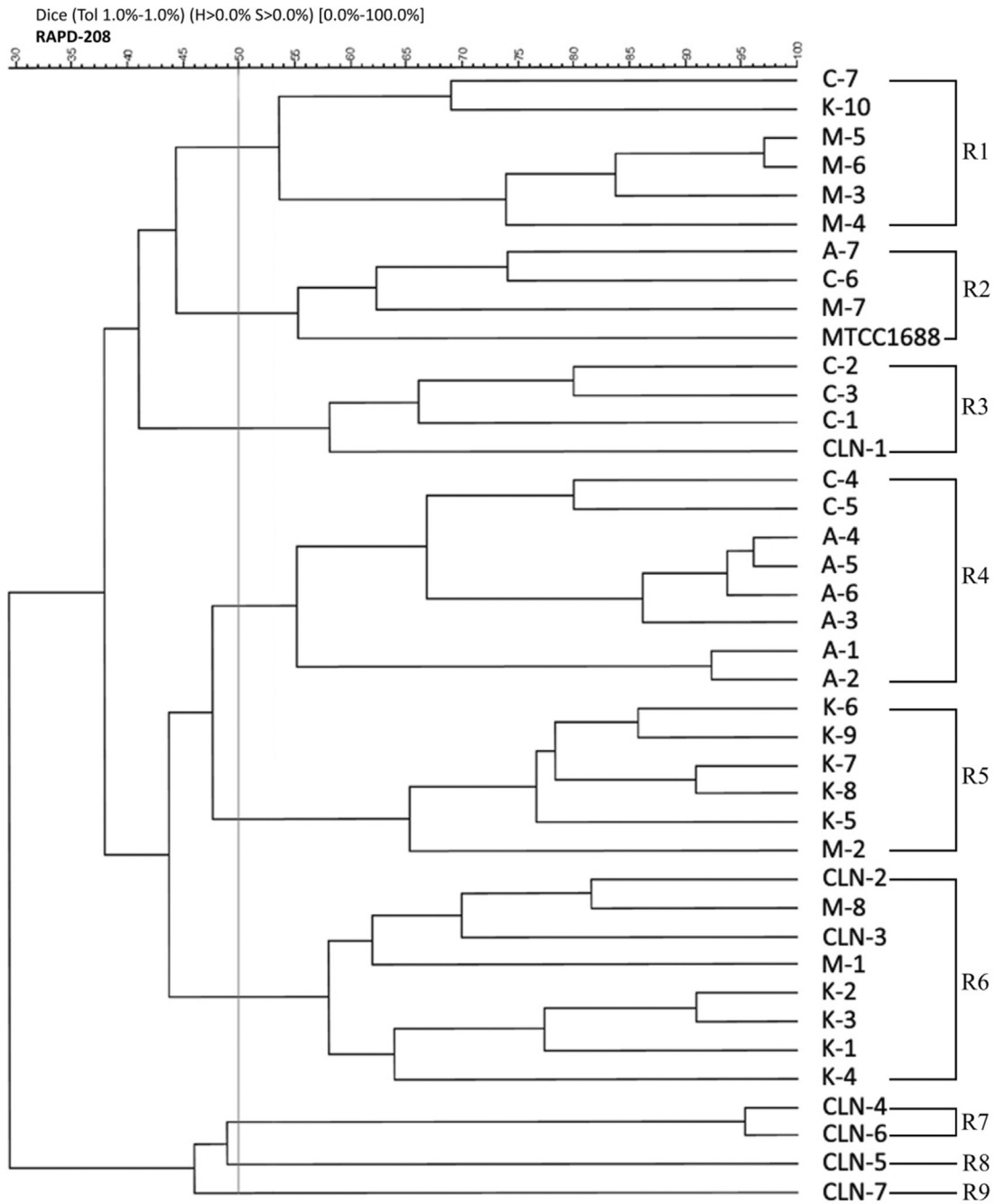
Figure 3 - An UPGMA dendrogram based on the AP-PCR banding patterns of the *P. aeruginosa* isolates (abbreviations as in Figure 2). R1-R9 are the clusters formed based on the RAPD pattern at the threshold level of 50% similarity.

antibiotics, except polymyxin and gentamicin, as compared to clinical isolates (Supplementary Figure 1).

The isolates from Andaman and Kakinada and the reference strain MTCC 1688 exhibited no significant differences (p > 0.05) in pyocyanin production and protease activity. However, pyocyanin production by the Cochin isolates (1.41 μg/mL) was significantly higher (p < 0.05) than that shown by the strains from the other locations (0.22-0.79 μg/mL). Significantly higher protease activity was observed in the isolates from Andaman and Kakinada (> 19 mm) compared with those from the other sampling stations (13 - 17 mm). The Mumbai isolates (21.88 mm) and the reference strain MTCC 1688 (20 mm) showed significantly higher (p < 0.05) haemolysin activity than the isolates from the other stations (11-17 mm). The isolates from both Andaman and Cochin exhibited similar haemolysin activity (11.14 mm) with no significant differences (Table 3).

Table 3 - Pyocyanin, Protease and Heamolysin activity of *P. aeruginosa* isolates collected from various stations.

| Station	Pyocyanin production (μg/mL)	Protease (zone in mm)	Haemolysin (zone in mm)
Andaman (n = 7)	$0.29^a \pm 0.02$	$20.43^a \pm 1.25$	$11.14^a \pm 0.52$
Cochin (n = 7)	$1.41^b \pm 0.08$	$13.57^b \pm 0.62$	$11.14^a \pm 0.80$
Kakinada (n = 10)	$0.24^a \pm 0.01$	$19.40^{ac} \pm 0.85$	$17.50^{bd} \pm 0.77$
Mumbai (n = 8)	$0.50^{ac} \pm 0.01$	$14.13^{bd} \pm 0.96$	$21.88^c \pm 1.06$
Clinical isolates (n = 7)	$0.79^c \pm 0.03$	$14.71^{bd} \pm 1.10$	$14.29^{ab} \pm 0.67$
Reference strain (n = 1)	$0.22^a \pm 0.01$	$17.00^{cd} \pm 0.13$	$20.00^{dc} \pm 0.32$

The data for each isolate are presented as the mean ± standard deviation. The column values with different letters (a-d) are significantly different ($p < 0.05$).

The dendrogram constructed based on these phenotypic traits (antibiotic susceptibility, haemolysin, pyocyanin, and protease production) showed that about 90% of the isolates had 75% similarity (Figure 4). Based on the similarity indices, most isolates showed high phenotypic relatedness in spite of their different geographical origin.

Discussion

Even though *P. aeruginosa* has high adaptability and thrives in a wide range of dissimilar ecosystems, the occurrence of the organism in coastal waters from the four locations in India was very low. A similarly low recovery of this species has also been reported earlier from the southeastern coastal waters of India (Nallathambi *et al.*, 2002; Mohandass and LokaBharathi, 2003) and from the waters of Japan (Khan *et al.*, 2007; Nonaka *et al.*, 2010). The low occurrence may be due to factors such as external stimuli and local competition (Stover *et al.*, 2000; Pirnay *et al.*, 2005). Since the coastal waters sampled in the present study received various pollutants and allochthonous bacteria, including coliforms (Mohandass and LokaBharathi, 2003; Ramaiah *et al.*, 2004), possible competition with other microbes could explain the low occurrence of *P. aeruginosa* in these waters (Vives-Flórez and Garnica, 2006). Besides, the scarcity of nutrients in marine waters may lead to the development of novel phenotypes which are better adapted to starvation conditions and unable to grow in the rich cultivation media used in laboratories (Kimata *et al.*, 2004; Khan *et al.*, 2007).

Our results did not show any clear-cut geographical or niche-based separation among the isolates. The FAME analysis did not reveal any geographical or origin specificity among the *P. aeruginosa* isolates, thus corroborating the net-like population structure of the species. Consistent with many previous studies, most clinical and environmental isolates were indistinguishable in their pathogenic characters, such as antibiotic sensitivity and pyocyanin, protease and haemolysin production (Alonso *et al.*, 1999; Finnan *et al.*, 2004; Vives-Flórez and Garnica, 2006). A few clinical and environmental isolates were even resistant to multiple antibiotics currently recommended as anti-pseudomonal
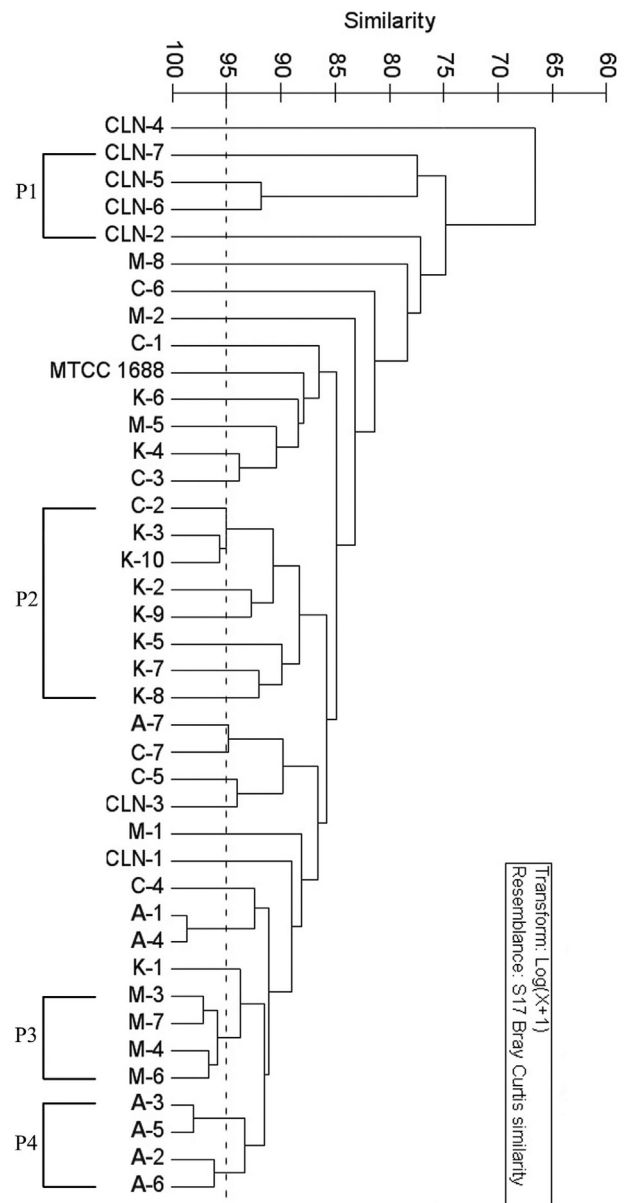
Figure 4 - Dendrogram based on the phenotypic patterns of the *P. aeruginosa* strains. The dendrogram was constructed using the PRIMER-5 software (abbreviations as in Figure 2). P1-P4 are the clusters formed at a similarity level above 95%.

drugs (Carmeli *et al.*, 1999). These resistance capabilities can be attributed to the intrinsic characteristics of *P. aeruginosa,* such as a low permeability of the cell wall, active efflux mechanisms, and the presence of resistance genes (Lambert, 2002). Additionally, *P. aeruginosa* is naturally resistant to many groups of antibiotics, including macrolides, chloramphenicol, fluoroquinolones, and β-lactams (Pirnay *et al.*, 2005). Its resistance to synthetic antibiotics, especially quinolones, is likely due to the multidrug efflux system (Alonso *et al.*, 1999). Similar multiple resistances have already been reported for strains from the open ocean by Khan *et al.* (2007). The large genome of *P. aeruginosa* (Stover *et al.*, 2000) is rich in regulatory genes and presumably reflects the fact that most strains encounter a broad range of ecological niches (Spiers *et al.*, 2000). This could be a probable reason for the uniformity in phenotypic expression.

On the contrary, AP-PCR showed some distinct clustering, owing to its ability to resolve clonal relationships with high reproducibility and discrimination power (Hernandez *et al.*, 1997). All but three clinical strains of *P. aeruginosa* formed a separate cluster that was distantly related to the environmental isolates. However, CLN-1 grouped with Cochin isolates, and CLN-2 and CLN-3 grouped with Mumbai isolates, suggesting that the environmental isolates might have had a clinical origin as these sites have been under excessive anthropogenic intervention. Similarly, clustering of clinical isolates with marine (Nonaka *et al.*, 2010) and terrestrial isolates (Römling *et al.*, 1994; Kiewitz and Tummler, 2000; Ruimy *et al.*, 2001) has already been reported. The occurrence of clinical isolates in coastal waters and their clustering with environmental isolates have become unavoidable as many coastal waters are not only influenced by fresh water and terrestrial environments, but also serve as a receptacle of hospital and domestic waste discharges (Khan *et al.*, 2008). In the present study, the uniqueness of the genotype was obvious for environmental isolates, especially marine, from sites with less anthropogenic intervention (Andaman), thus confirming the observation of Khan *et al.* (2008) that marine isolates are distinct from clinical isolates.

To our knowledge, this is the first attempt to understand the influence of the environment on genetic, metabolic and phenotypic characteristics of the same set of *P. aeruginosa* strains isolated from coastal and marine waters of India*.* The isolates from Mumbai and Cochin were more scattered, suggesting that the prevalence of *P. aeruginosa* was considerably influenced by the anthropogenic effects on these particular ecosystems. Since the sites of water collection in Cochin and Mumbai were close to the port areas, it is possible that transportation of non-indigenous strains through the ballast water could have led to the diverse clusters (Ramaiah *et al.*, 2004). The lack of consistency in cluster formation may be due to the high frequency of recombinations and easy adaptation observed in *P. aeruginosa*

(Pirnay *et al.*, 2005; Nonaka *et al.*, 2010). Since the isolates from the most polluted Mumbai region were even more scattered compared to those from the Cochin region, the genotypic variability seems to be influenced by the extent of pollution as well. In any case, the distribution of clinical strains in the environment increases the risk for individuals and also affects the eco-balance.

Conclusion

The effect of ecosystem on genetic, metabolic and phenotypic characteristics of *P. aeruginosa* isolates from Indian coastal waters was studied. Cluster-based similarity analysis showed that the prevalence of *P. aeruginosa* was considerably influenced by the anthropogenic effects on the ecosystem as the isolates from Mumbai and Cochin were more scattered. However, genetic characterisation using AP-PCR helped to separate isolates of marine origin and revealed their homogeneity and also separated isolates according to the extent of pollution. A detailed study using newer methods is required to confirm whether the differences in the genetic fingerprints between the clinical and environmental isolates observed in this study are indeed reflected at the genome/transcriptome level. Thus, it appears that phenotypic and metabolic characteristics are not exclusive criteria for differentiation of environmental and clinical strains.

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Supplementary Material

Figure S1. Antibiotype of *P. aeruginosa* strains. Clinical strains include a reference strain (MTCC1688) also.
Table S1: Details of the primers used in the study.
Table S2: Relative occurrences of fatty acid in each ecotype.

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