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PSEUDOMONAS AERUGINOSA ASSOCIATED WITH NEGATIVE INTERACTIONS ON COLIFORM BACTERIA GROWTH

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ABSTRACT

Screening aquatic Pseudomonads which inhibit growth of *Escherichia coli* and *Enterobacter aerogenes* produced sixteen strains of pyocyanin-producing *Pseudomonas aeruginosa.* Cell enumeration was carried out by the Most-Probable-Number technique for 96h by using diluted Müeller-Hinton broth. Conditions favouring pyocyanin production resulted in reduced growth of coliform strains. Representative strains were resistant to ciprofloxacin, norfloxacin and imipenem. It was also verified that a high population of coliform strains was reached when grown individually. Results show that there was an antagonistic phenomenon provided by *Pseudomonas aeruginosa* against coliform bacteria when pyocyanin was formed. This paper highlights the risks for human and environmental health that this phenomenon represents.

Keywords: Pyocyanin-producing *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter aerogenes*, antagonism.

INTRODUCTION

Pseudomonas aeruginosa (Migula, 1894) is a formidable widespread organism occurring naturally in water and other environments (Trivedi *et al*., 2008; Shrivastava *et al*., 2004). However it is most often described as an opportunistic pathogen involved in nosocomial infections and episodic outbreaks (Bou *et al*., 2009; Corvec *et al*., 2008).

Pyocyanin (phenazine 5-methyl-1-hydroxy phenazinium betaine) is a pigment produced by over 90% of strains (Mavrodi *et al*., 2001; Reyes *et al*., 1981) and some authors suggest that pyocyanin is particularly responsible for the antagonistic phenomenon against other microorganisms through the generation of reactive oxygen species which would be expected to exert antimicrobial activity toward coliform bacteria (D'áquila *et al*., 2000; Guilherme and Silva, 2000; Duncan *et al*., 1999).

The coliform bacteria comprise more than 30 taxonomically different microorganisms. Genera *Escherichia* and *Enterobacter* are among the most important of the group. Since the 19th century, coliform bacteria have been considered effective indicators of recent fecal pollution in water and *E. coli* (Escherich, 1895) is the most important microorganism from this point of view (Foppen and Schijven, 2006).

There are several common laboratory methods used for estimating concentration of viable cells. Most-Probable-Number (MPN) is a rapid and simple method and important reasons for choosing this method can be listed: 1- when replacing a solid medium method due to the kinetic of growth of microorganisms that are highly variable and would obscure colonies of the organism of interest; 2- It is useful when microorganisms of interest produce some detectable product and cells can be estimated; 3- solid media may have factors such as heavy metals that alter the reliability of the count or interfere with the aim of the experimental plan and, 4- even if contaminating organisms overgrow the culture, the cells of interest can still be estimated (Gerhardt *et al*., 1994).

Coliform enumeration may be affected by several physical and chemical sources such as temperature, pH, chloride content, salinity and metals which could mask the analysis leading to false-negative results. In addition, biological agents also interfere by inhibiting coliform cell growth through various mechanisms which includes amensalism, pigment concentration and competition for nutrients between non-coliforms and coliforms (Spangenberg, 2007).

Several microorganisms may be antagonistic to the coliform group. Due to its opportunistic nature and metabolic versatility, *P. aeruginosa* strains are one of most important injury-promoting species of coliform bacteria inhibition in aquatic environments (Tamagnini and Gonzáles, 1997). Earlier data on negative interactions toward *E. coli* in water analysis identified some genera other than *Pseudomonas* may also be involved in this antagonistic phenomenon, such as Gram-positive

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Actinomyces, *Micrococcus* and *Sarcina*, Gram-negative *Flavobacterium* and yeasts (Hutchinson *et al*., 1943).

This paper reports a negative interaction of *P. aeruginosa* strains isolated from different aquatic sources against coliforms *E. coli* and *E. aerogenes* (Kruse, 1896). Tests were conducted to detect this phenomenon by measuring population of *P. aeruginosa* and coliform bacteria incubated simultaneously by simulating a recent coliform contamination in an environment where pyocyanin-producing *P. aeruginosa* strains were already established.

MATERIALS AND METHODS

Bacterial strains, media and identification methods

A total of sixteen aquatic strains of *P. aeruginosa* were investigated in this study. All strains were isolated from environmental and clinical water samples surrounding Recife, capital of the state of Pernambuco, Brazil and data are shown in Table 1. Representative *E. coli* EC-5 and *E. aerogenes* EA-1, respectively, were isolated from distinct well water samples where *P. aeruginosa* was absent. The reference type strains of *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *E. aerogenes* ATCC 15012 were also included in the study.

All *P. aeruginosa* strains were identified by using asparagine and acetamide broth at 37±2°C for 24-48h of incubation, fluorescence at 360±20nm wavelength, growth on cetrimide Agar (Acumedia, Maryland, US) and enzymatic oxidase test (Probac do Brasil, São Paulo, Brazil). In order to guarantee conditions favouring the enhancement of pyocyanin, *P. aeruginosa* strains were grown on Pseudomonas P medium. During the tests, the presence of pyocyanin was verified by the discoloration of acidic $KMnO_4$ solution and by observing fluorescence at 360±20nm (Health Protection Agency, 2003; APHA *et al*., 1998).

E. coli and *E. aerogenes* strains were identified by using lactose broth and green brilliant bile broth at 37±2°C for 24-48h of incubation, and confirmed in EC broth and eosin-methylene-blue agar, respectively. Biochemical tests: indole production, methyl-red test, Vokes-Proskauer test and citrate utilization, were also performed (APHA *et al*., 1998).

Antibiotic sensitivity was assessed using Müeller-Hinton agar (Merk, Darmstadt, Germany) and antibiotic disks (Sensidisc-DME, São Paulo, Brazil).

Characterization of strains inhibitory to coliform

The assay was conducted as described by Ichikawa *et al*. (1971). *P. aeruginosa* strains were subcultured onto the surface of Müeller-Hinton agar disks and incubated in sterilized Petri dishes at 37±2°C for 48h. Then, coliforms were spread on the surface of Müeller-Hinton agar and disks containing grown *P. aeruginosa* were transferred onto the agar surface and incubated at 37±1°C overnight. Tests were carried out in triplicate. The inhibition zones formed were measured. A greater inhibition zone diameter indicated more coliform susceptibility to *P. aeruginosa*.

Table 1. The aquatic sources of *Pseudomonas aeruginosa* strains.

Strains	Source	Counts (MPN.100ml^{-1})	Multi-drug resistance (from 8 antibiotics tested)
PA1	Várzea Cemitery (well #1)	$\geq$ 1600	0
PA2	Várzea Cemitery (well #2)	$17x10^3$	2
PA3	Várzea Cemitery (well #3)	$\geq$ 1600	0
PA4	Tap (hospital)	$7x10^3$	0
PA5	Bottled water	$26x10^3$	0
PA6	Storage tank (hospital)	$17x10^4$	0
PA7	Well (domestic)	$11x10^2$	0
PA8	Water cooler (school)	ND	0
PA9	Tap (school)	ND	0
PA10	Tap (school's kitchen)	ND	0
PA11	Tap (kinder care's kitchen)	ND	0
PA12	Tap (hospital)	$23x10^4$	0
PA13	Tap (UFPE campus bathroom)	ND	0
PA14	Landfill (influent tank)	$6x10^5$	3
PA15	Landfill (facultative lagoon)	$2x10^4$	0
PA16	Well (industry plant)	ND	3

ND – not determined. Strain was isolated without quantification

Inhibitory activity test in liquid-state

The three most harmful strains in the selective inhibitory activity assay on solid medium were studied using diluted Müeller-Hinton Broth (Vetec, Rio de Janeiro, Brazil). The assay comprised the *P. aeruginosa* and coliform enumeration of the Most-Probable-Number for a period of 96h at intervals of 24h by using the multiple tube technique with 5 tubes per dilution: 10.0, 1.0 and 0.1mL. A sample from *P. aeruginosa* suspension (around 10^2 CFU/mL) was inoculated and incubated at 37±1°C for 72h, after which either EA-1 or EC-5 inoculation occurred by transferring their suspensions (around 10^2 CFU/mL) into the flask and re-incubating overnight. Tests were also conducted between reference type strains. Control tests with each strain grown individually were also carried out using the same conditions and time intervals.

RESULTS AND DISCUSSION

In this study we investigated the antagonistic interactions among pyocyanin-producing *P. aeruginosa* strains characterized by *in vitro* assays on solid and in liquid medium where the coliform bacteria represented a recent contamination in an environment where *P. aeruginosa* has already been established.

All *P. aeruginosa* strains previously confirmed their ability to produce pyocyanin and further showed antimicrobial activity against coliform bacteria, detected by measuring the inhibition zone diameters formed. Fluorescein production was also observed in five strains. Diameters from 9 to 17±0.1mm for EC-5 and from 7 to 29±0.1mm for EA-1 ($p<0.05$) were found and are summarized in Table 2.

Based on the inhibition zone size between reference type strains in the inhibitory activity assay performed on solid medium, six *P. aeruginosa* strains formed inhibition zones less than 11±0.1 mm and nine reached values more than 11±0.1 mm against EC-5 ($p<0.05$). Against EA-1, seven *P. aeruginosa* strains showed inhibition zone sizes of more than 16±0.1 mm and eight less than 16±0.1 mm ($p<0.05$). *P. aeruginosa* archived similar inhibition patterns for both coliforms despite the differences in inhibition zone sizes. Results based on the characterization of inhibitory activity were important for choosing the most suitable strains for the inhibitory activity test in liquid-state. It was established that the greater antimicrobial activity against *E. coli* would be tested further. Then, PA2, PA14 and PA16 were selected for the following investigation.

After the contact between the strains in liquid medium, the interval between 24 and 48h was decisive for *P. aeruginosa* to be successful. While coliform bacteria viable cells started to decay in number, *P. aeruginosa* strains increased and maintained their growth until 96h. Legnani *et al.* (1999) concluded in their study that when competing for nutrients, *P. aeruginosa* showed a faster initial growth within 48h, then, growth slowed in order to allow log phase to continue up to 7 days. Similar findings were also observed in the present study.

The percentage of reduction in number of coliform cells is

Table 2. Growth inhibition of coliform bacteria strains by *Pseudomonas aeruginosa* and by reference strain differing in pigment production.

P. aeruginosa strains	Pigment	Indicator coliform bacteria strains (inhibition zones)	
		Enterobacter aerogenes EA-1	*Escherichia coli* EC-5
PA1	Pyocyanin, Fluorescein	+	++
PA2	Pyocyanin	+++	+++
PA3	Pyocyanin, Fluorescein	+	++
PA4	Pyocyanin, Fluorescein	++	++
PA5	Pyocyanin	+++	++
PA6	Pyocyanin, Fluorescein	++	+
PA7	Pyocyanin	+	+
PA8	Pyocyanin	+++	++
PA9	Pyocyanin, Fluorescein	+	++
PA10	Pyocyanin	+++	++
PA11	Pyocyanin	+++	++
PA12	Pyocyanin	+	++
PA13	Pyocyanin	++	++
PA14	Pyocyanin	+++	+++
PA15	Pyocyanin	+++	++
PA16	Pyocyanin	++	+++

shown in figure 1. Among wild-type strains, reduction in number of cells ranged 18.3-29.8±0.1% for EA-1 and from 9.8 to 40.3±0.1% for EC-1 ($p<0.05$). Although the two coliform bacteria presented similar inhibition patterns in the presence of PA2 (21.3 and 21.1±0.1% for EA-1 and EC-5, respectively), they differed among the other *P. aeruginosa* strains in terms of inhibitory activity by developing a pronounced susceptibility toward PA14 and less susceptibility toward PA16. Many reasons may be hypothesized in order to elucidate these results, such as, pigment concentration or multi-drug resistance. In addition, environmental selective pressure found at isolation sites may explain the inhibitory pattern of the strains.

Disturbed environments such as leachate-contaminated waters tend to permit physiological evolution and increase of gene pool by mutation as well as limited selective pressure. In addition, genetic exchange between microorganisms and external stress may act as driving forces on microbial size and stability (Evans *et al*., 1981). To know that PA14 and PA16 strains were isolated from landfill's influent tank and industry well, respectively, it is clearly obvious that environmental stress exerted influence on pyocyanin-producing *P. aeruginosa* strains metabolic versatility and also may have contributed most to the antimicrobial activity toward coliforms.

This affirmation may be reinforced with results from the control test. Among type-strains, *E. aerogenes* proved to be more sensitive than *E. coli* toward type-strain *P. aeruginosa*. Selective pressure would be related to driving forces in order to enhance metabolic versatility and may permit *P. aeruginosa* to present antagonistic antimicrobial activity against non-caustic environmental isolates. The two type-strains *P. aeruginosa* and *E. coli* were originated from nosocomial environments and this would explain why *E. coli* showed more resistance than wild type-strain *E. aerogenes.*

In figures 2 and 3, inhibitory antagonistic phenomenon between EA-1 and EC-5 and pyocyanin-producing *P. aeruginosa* strains is detailed as the development of coliforms cell (increase and decrease) within 96h at intervals of 24h in the liquid-state test and for the control, i.e., EA-1 and EC-5 individually grown in diluted Müeller-Hinton broth.

Presence of pyocyanin-producing *P. aeruginosa* strains strongly disturbed the two coliforms' growth when they were inoculated into tubes of diluted Müeller-Hinton broth simultaneously. A decreasing of growth pattern was observed, and the first 24-48h was decisive for the event, possibly related to the physiological effects of injury stress during the coliforms' lag time. At the end of the assay, major decrease in growth of EA-1 and EC-5 (against PA14) reached 3 orders of magnitude. Moreover, reduction in growth has not been observed in control tubes and led us to confirm that pyocyanin-producing *P. aeruginosa* strains have acted as a biological interference on coliform analysis. We emphasize that though coliform bacteria were reduced in number of estimated cells in the present study, they still reached significant levels.

In order to elucidate if the detected antagonistic interaction corresponded to a bacteriostatic or bactericidal event, plating of serial dilutions of the coliform suspensions at time 0h and a sample from the tube 96h later revealed EA-1 and EC-5 to be recovered on eosin-methylene-blue agar. Furthermore, the number of colony forming units was apparently reduced by the presence of the antagonist.

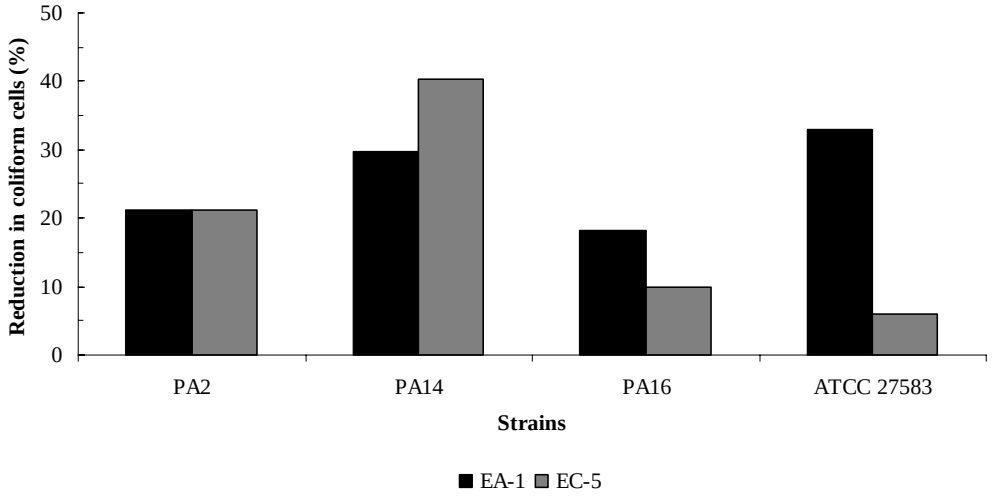

Fig. 1. Decrease in coliform growth after 96h.

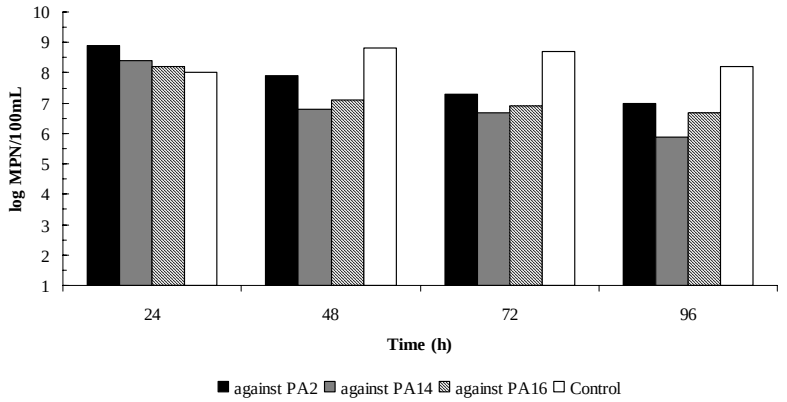

Fig. 2. Patterns of EA-1 growth inhibition in the presence of pyocyanin-producing *Pseudomonas aeruginosa* strains. Control refers to EA-1 grown individually. Single values from experiments repeated on at least two separate occasions.

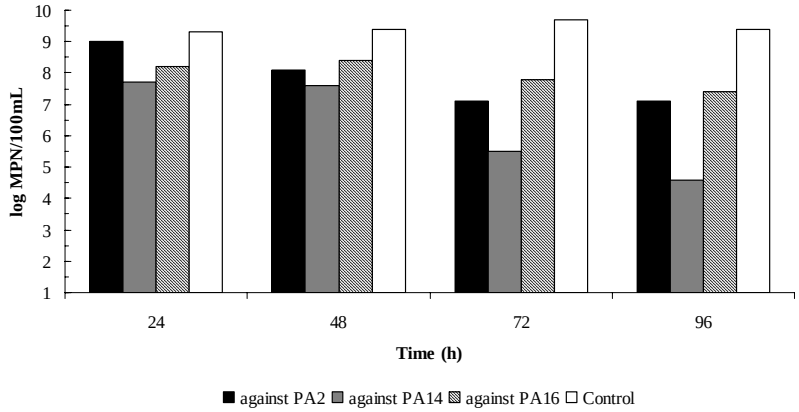

Fig. 3. Patterns of EC-5 growth inhibition in the presence of pyocyanin-producing *Pseudomonas aeruginosa* strains. Control refers to EC-5 grown individually. Single values from experiments repeated on at least two separate occasions.

Our findings were consistent to a classical study on the incidence and significance of microorganisms antagonistic to the coliform group which demonstrated a reduction of 28-97% when antagonist and *E. coli* were inoculated simultaneously (Hutchison *et al*., 1943). The lower reduction in percentage of coliforms found in this study was possibly caused by the different culture medium and initial cell concentrations used.

The use of diluted medium stimulated competition for carbon sources where coliform bacteria and *P. aeruginosa* were forced to overlap. Technically, coliform bacteria would have advantages due to shorter doubling-time when compared to *P. aeruginosa* (Tamagnani and Gonzáles, 1997; Camper *et al*., 1991). Nevertheless, pyocyanin-producing *P. aeruginosa* strains have proven to be more nutritionally versatile and capable of adaptation when competing with another microorganism. Moreover, literature reported that under competition for nutrients, coliform bacteria usually delay in lag time which would also explain the susceptibility of that group in the present study (Evans *et al*., 1981).

Although coliform bacteria are the universal indicators of fecal pollution in waters for human consumption, detection of *P. aeruginosa* as a complementary test is matter of concern because presence of *P. aeruginosa* and low coliform bacteria concentration would hide a major risk and would compromise the results from current methods used for water analysis.

P. aeruginosa creates an element of concern, as this species can cause nosocomial infections or multi-drug resistant strains which may be untreatable. The three most harmful strains in this study were also resistant to at least three important antibiotics used for the treatment of *P. aeruginosa* infections, as follows (concentration tested in $\mu g.ml^{-1}$): ciprofloxacine (5), norfloxacine (10) and imipenem (10), and were sensitive to amikacin (30), ceftazidime (30), gentamicin (10), ticarciline/clavulanate

(75/10) and tobramycin (10). The other thirteen remaining strains were sensitive to all antibiotics tested. Multi-drug resistance may also be related to selective pressures exerted by the environment influencing physiological evolution as discussed early.

Some authors have also discussed that *P. aeruginosa* tends to persist in water for a longer period of time and it is metabolically able to resist caustic agents in nature, as opposed to coliform survival which decreases with sunlight and allows opportunistic pathogens to grow (Schultz-Fademrecht *et al*., 2008; Ahlén *et al*., 1998). Guilherme and Silva (2000) affirmed that pyocyanin is accepted as the main factor in the antagonistic role of *P. aeruginosa*. Other mechanisms of action of pyocyanin have been discussed in literature, which include involvement with the redox reaction and formation of superoxide and hydrogen peroxide with further ATP depletion (O'Malley *et al*., 2003; Denning *et al*., 1998).

The *P. aeruginosa* bacteriocins suggest a potential role in environmental ecology, and further studies must be carried out to evaluate the effect of pyocyanin concentration in the process of coliform inhibition. The presence of pyocyanin-producing *P. aeruginosa* strains themselves would lead to inhibitory activity against coliform bacteria. If such microorganisms belong to the feacal coliform group, great attention must be given due to their epidemiological importance.

Reduction in coliform growth induced by *P. aeruginosa* may lead to situations where the drinking water would be considered potable, however, under favorable conditions, coliform re-growth may develop and interspecies equilibrium would re-establish within the environment. Hence, coliform bacteria activation even in the presence of Psedomonads may possibly portray a hazardous scenario and may lead to real human and environmental health risks.

This study also provided some contribution to the knowledge of the action of pyocyanin-producing *P. aeruginosa* related to environmental control of coliform bacteria. Although our findings do not represent a general rule and site specific studies are needed, the methodological approach used here can be a relevant support tool when designing water analysis strategies.

CONCLUSIONS

The results of the experimental work undertaken in the present study described one inhibitory activity of *P. aeruginosa* against coliform bacteria in solid and liquid media. This effect could lead to human and environmental health risks if, for example, total coliform concentrations in water samples were low or not detectable as a result of the presence of *P. aeruginosa*, but then subsequent favorable conditions allow for coliform re-growth, including potentially pathogenic strains.

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