Influence of hydrocarbons on the virulence and antibiotic sensitivity associated with
*Pseudomonas aeruginosa*

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The bacterium, *Pseudomonas aeruginosa* has the potential for use in bioremediation. However, this organism is an opportunistic pathogen and is highly resistant to disinfectants and antibiotics. *P. aeruginosa* has been known to cause a wide variety of infections in humans, especially in immunocompromised persons. The purpose of this study was to assess the influence of hydrocarbons on the virulence of *P. aeruginosa* as it degrades these hydrocarbons. The expression of virulence factors associated with 12 different hydrocarbon-degrading strains of *P. aeruginosa* was evaluated. Virulence factors including hemolytic activity, antibiotic sensitivity, cell adherence, and exopolysaccharide capsule formation were tested in the presence of 0.1% glucose and the appropriate hydrocarbon. No differences were found in hemolytic activity or antibiotic resistance in the presence of glucose or hydrocarbon. Growth on glucose significantly enhanced adherence, while growth on a hydrocarbon enhanced capsule formation. The results of this study indicate that, overall, growth in the presence of hydrocarbons such as hexadecane does not enhance the virulence characteristics of *P. aeruginosa*.

*Keywords*: *seudomonas aeruginosa*; virulence factors; biodegradation; hydrocarbons.

**Introduction**

There is a strong and continuing interest in removing or detoxifying organic pollutants in the environment. An emerging approach is the use of microorganisms, including *Pseudomonas aeruginosa*, to degrade these contaminants. *P. aeruginosa* is capable of degrading a wide variety of organic compounds. Studies have shown that *P. aeruginosa* can degrade gasoline products (Al-Hadhrami et al. 1995), mono- and polychlorinated benzoic acids (Hickey and Focht 1990), bromo benzoic acids (Higson and Focht, 1990), acetonitrile (Chapatwala et al. 1990), a number of nitriles and amides (Aislabie and Atlas 1988) and can also enhance the mineralization of polychlorinated biphenyls (Hickey et al. 1993). Even wool grease (found in wool scour effluent), which is recalcitrant to degradation, can be degraded by a strain of this organism (Brahimi-Horn et al. 1991). In addition, recent studies have reported that extracellular biosurfactants produced by this bacterium play a role in solubilizing and dispersing hydrocarbons (e.g. petroleum products) in soil and enhancing their removal (Jain et al. 1992, Scheibenbogen et al. 1994, Providenti et al. 1995). Therefore, *P. aeruginosa* has a potential for use in bioremediation (Al-Hadhrami et al. 1995, Providenti et al. 1995).

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However, in the past two decades, *P. aeruginosa* has come forth as an important opportunistic pathogen. *P. aeruginosa* has an array of virulence factors which are related to serious infections in humans, especially in immunocompromised individuals (Bodey et al. 1983, Pollack 1990). Some of the virulence factors include production of exotoxin A, phospholipase C, proteases, pili, exopolysaccharide capsule, and pigments (Bodey et al. 1983).

In the present study, 12 different hydrocarbon-degrading strains of *P. aeruginosa* were examined for the presence of virulence factors. The strains were grown on both glucose and the hydrocarbon that they degrade, and the extent of virulence expressed was compared and evaluated. The majority of the strains used degraded the hydrocarbon hexadecane. The strains grown on glucose served as a control and represented *P. aeruginosa* found in nature such as in soil and water uncontaminated by hydrocarbons, and on plants. The virulence factors examined included hemolytic activity, cell adherence, exopolysaccharide capsule formation as well as antibiotic sensitivity which could also be considered a virulence factor as it renders the microorganism resistant to treatment.

**Materials and methods**

**Bacterial strains**

Twelve different hydrocarbon-degrading strains of *P. aeruginosa* were used in this study (Table 1) including: JB2 supplied courtesy of Dr D. Focht, University of California, Riverside; UG2 was kindly provided by Dr H. Lee, University of Guelph, Canada; KDC was supplied courtesy of Dr K. Chapatwala, Selma University, four of the seven strains (ATCC 27853, ATCC 15442, ATCC 9027 & NRRL 3198) were supplied courtesy of Dr R. Miller, University of Arizona. The remainder of the strains were obtained from the American Type Culture Collection (Rockville, MD). Two additional strains were used. One (CF isolate) that was multiple-antibiotic-resistant and demonstrated capsular formation had been isolated from a cystic fibrosis patient. The other was a multiple-antibiotic-sensitive quality control strain (QC, ATCC 27853). Both of these latter two strains were obtained from the University Medical Center, Tucson, Arizona.

**Table 1. *P. aeruginosa* strains and hydrocarbon used as sole carbon source**

<table>
<thead>
<tr>
<th>Strain of <em>P. aeruginosa</em></th>
<th>Degradable hydrocarbon</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 31479</td>
<td>Hexadecane</td>
<td>Soil: removal of oleaginous material from waste water</td>
</tr>
<tr>
<td>ATCC 15442</td>
<td>Hexadecane</td>
<td>Animal room water bottle</td>
</tr>
<tr>
<td>ATCC 9027</td>
<td>Hexadecane</td>
<td>Outer ear infection</td>
</tr>
<tr>
<td>NRRL 3198</td>
<td>Hexadecane</td>
<td>Unavailable</td>
</tr>
<tr>
<td>JB2</td>
<td>2-Chlorobenzoxide</td>
<td>Polychlorinated biphenyl-contaminated soil</td>
</tr>
<tr>
<td>ATCC 17423</td>
<td>Hexadecane</td>
<td>Hydrocarbon enrichment of soil</td>
</tr>
<tr>
<td>ATCC 27853</td>
<td>Hexadecane</td>
<td>Blood culture</td>
</tr>
<tr>
<td>ATCC 19154</td>
<td>Lanolin</td>
<td>Unavailable</td>
</tr>
<tr>
<td>ATCC 21472</td>
<td>Hexadecane</td>
<td>Soil from oil field</td>
</tr>
<tr>
<td>ATCC 15524</td>
<td>Hexadecane</td>
<td>Soil: utilizing n-alkanes, cinnamic acid and 5-phenyl valeric acid</td>
</tr>
<tr>
<td>UG2</td>
<td>Hexadecane</td>
<td>Soil</td>
</tr>
<tr>
<td>KDC</td>
<td>Acetonitrile</td>
<td>Soil and water samples near industrial areas</td>
</tr>
</tbody>
</table>
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\textbf{Culture conditions}

Each strain was maintained on a minimal mineral salts media (Bushnell Haas) containing (in g L\(^{-1}\)): MgSO\(_4\), 0.2; CaCl\(_2\), 0.02; KH\(_2\)PO\(_4\), 1.0; K\(_2\)HPO\(_4\), 1.0; FeCl\(_3\), 0.05; NH\(_4\)Cl, 1.5. For each strain, the medium was amended with the appropriate hydrocarbon or glucose as follows. 0.1% glucose (pH 7.2), 0.1% 2-chlorobenzoate (neutralized with 1 M NaOH), 0.1% acetonitrile (pH 6.7), 0.1% lanolin (pH 7.4), 0.5% hexadecane (pH 7.2). Both acetonitrile and 2-chlorobenzoate were filter sterilized and aseptically added to the respective medium. Lanolin and hexadecane were added to their respective medium and then autoclaved.

\textbf{Chemicals}

Hexadecane, 2-chlorobenzoic acid and acetonitrile were purchased from Aldrich Chemical Co Inc, Milwaukee, WI. Lanolin was obtained from Sigma Chemical Co., St. Louis, MO.

\textbf{Antibiotic resistance test}

The Kirby-Bauer disk diffusion test was used to determine antibiotic susceptibility (Bauer \textit{et al.} 1966). The following 10 antibiotics were used: ticarcillin, mezlocillin, amikacin, cefotaxime, azlocillin, tobramycin, piperacillin, carbenicillin, gentamicin, and ceftazidime. The antibiotics were purchased from Baxter Diagnostics, Hayward, CA. The quality control (QC) strain was used to fulfill requirements of the National Committee for Clinical Laboratory Standards (NCCLS, 1991). Zones of inhibition were interpreted according to the guidelines established by NCCLS.

\textbf{Hemolytic activity test}

Liu (1957) first demonstrated the use of cellophane plates to collect hemolysin. In this study, hemolytic activity was determined as described by Fujita \textit{et al.} (1988) with some modifications. The strains were grown on the surface of a dialysis membrane (mw 6000–8000) overlaying Bushnell Haas agar amended with glucose or a hydrocarbon as a sole carbon source. The membranes were inoculated with the appropriate strain and incubated at 37°C for 5 days. Hemolysin was obtained as follows: the lawn of bacterial growth was washed from the membrane surface with sterile distilled water and the bacterial suspension was adjusted to a target optical density of \(A_{600} = 1.13\) (cm\(^{-1}\)). The suspension was centrifuged at 3300 \( \times \) g for 25 min. Hemolytic activity was determined by making two-fold dilutions of the supernatant in phosphate buffered saline (PBS, pH 5.94) and adding 1% sheep red blood cells (which had been washed three times with physiological saline). The sheep red blood cells were purchased from Microbio Products, Inc., Tempe, AZ. The suspensions were incubated for 1 h at 37°C and then centrifuged at 600 \( \times \) g for 5 min. The two controls used in this test included a negative control comprised of 1% sheep red blood cells in PBS, and a positive control prepared by repeated freezing and thawing of the sheep red blood cells. Serial two-fold dilutions were made of the lysed red blood cells. The extent of hemolysis was determined by measuring the absorbance of liberated hemoglobin at 570 nm. All tests were performed in duplicate.

\textbf{Cell adherence test}

The bacterial cultures were grown on Bushnell Haas medium amended with glucose or a hydrocarbon as a sole carbon source for 5 days at 37°C. The cultures were suspended in 0.85% sterile saline to a target optical density of \(A_{600} = 0.23\) (cm\(^{-1}\)). Cell adherence assays were carried out on a continuous cell line; Buffalo Green Monkey (BGM) kidney cells. The BGM cells were grown in minal essential medium (MEM) (Flow Laboratories, McLean, VA)
containing 5% fetal bovine serum and propagated in six-well trays. The confluent monolayers of BGM kidney cells were washed twice with tris (hydroxymethyl amino-methane) (Sigma), pH 7.4, reagent-grade 99.9%, to remove the maintenance media. Each well was inoculated with 1 mL of the respective bacterial suspension. The trays were incubated for 1 h at 37°C on a Gyrotory Incubator Shaker rotating at 50 rpm. The cells were washed gently six times with tris buffer to remove any nonadherent bacteria. To each well, 4.0 mL of sterile distilled water were added in order to lyse the monolayer (BGM kidney cells). The trays were incubated at room temperature for 2 h. When the monolayer was lysed, the bacterial suspension was removed and put into a centrifuge tube containing sterile glass beads. The tubes were vortexed and bacterial enumerations were performed using the spread plate method on Tryptic Soy agar (TSA, Difco Laboratories, Detroit, MI). The spread plates were incubated at 37°C for 24 h, thereafter colony forming units (CFUs) were counted on each plate. The number of tissue cells in the monolayer were determined by using (0.05%) trypsin-EDTA (Gibco BRL, Grand Island, NY) to remove the monolayer. The action of trypsin was stopped by adding MEM. The cells were centrifuged, resuspended in MEM and vortexed gently to break any clumps. The cells were then counted using a hemocytometer. The results were then expressed as a ratio of bacterial cells per tissue cell. The tests were performed in duplicate.

Capsule formation
Capsule formation can be determined using several methods such as the Duguid staining method (Duguid 1951), Anthony staining method (Anthony 1931), and a dry-film method using Congo Red and Maneval’s solution. However, in this study satisfactory results were not achieved using any of these methods. These methods were successful with bacteria such as Klebsiella but proved to be inapplicable in the case of P. aeruginosa. For this study, capsule formation was determined through visual judgment. The bacterial strains were grown on the appropriate medium for 1 week. Colonies formed were examined to assess whether they were mucoid or nonmucoid (smooth or rough appearing). Visual judgment for capsule formation was based on the mucoid appearance of the CF isolate.

Results
Antibiotic disk diffusion
Twelve different hydrocarbon-degrading strains of P. aeruginosa were studied to investigate their susceptibility to 10 different antibiotics. A few differences were observed in the antibiotic sensitivity for P. aeruginosa strains pre-grown on glucose or a hydrocarbon (Table 2). Most of the strains were multiple-antibiotic-sensitive. Results showed that 89% of the strains grown on glucose and 92.5% of the strains grown on the hydrocarbon were sensitive to the antibiotics tested. Some of the noticeable differences were (1) strain KDC was resistant to carbenicillin when pre-grown on acetonitrile, and carbenicillin-sensitive when pre-grown on glucose; (2) strain ATCC 17423 was resistant to mezlocillin when pre-grown on hexadecane and sensitive when pre-grown on glucose; and (3) some of the strains were more resistant to cefotaxime when grown on glucose. In this study, zones of inhibition interpreted as intermediate by NCCLS criteria, were categorized as resistant.

Hemolytic activity
To objectively determine the extent of hemolytic activity expressed by each strain, a spectrophotometer was used to measure the absorbance of liberated hemoglobin. Hemolytic
activity ratio was defined as the absorbance of hemoglobin liberated by each strain divided by absorbance of the control. The results indicate that all the strains expressed hemolytic activity to some degree (Fig. 1). Strain ATCC 15442 expressed the greatest degree of hemolytic activity resulting in the highest absorbance ratio when pre-grown on the hydrocarbon. Analysis of variance showed that there was no significant difference in the hemolytic activity between the strains pre-grown on glucose or the hydrocarbon (p = 0.899).

**Cell adherence**

Figure 2 illustrates the adherence properties of the *P. aeruginosa* strains after pre-growth on glucose or the appropriate hydrocarbon. The results are expressed as a ratio of the CFU of bacteria adhered per tissue cell (BGM kidney cells). The strains pre-grown on glucose were significantly more adherent (p = 0.018) than the hydrocarbon-grown strains. Only 2 of the 12 test strains (ATCC 31479 and KDC) demonstrated enhanced adherence after growth on a hydrocarbon. When pre-grown on glucose, strain ATCC 15524 demonstrated the greatest adherence, while strain ATCC 21472 was more adherent than any other strain after pre-growth on a hydrocarbon.

**Capsule formation**

In order to evaluate capsular formation, the appearance of *P. aeruginosa* colonies grown on the appropriate hydrocarbon and glucose were examined. Only 17% of the strains grown on glucose formed capsules compared to 83% of the strains grown on a hydrocarbon (Table 3). Encapsulated strains produce smooth mucoid colonies while non-encapsulated strains form rough colonies.

**Discussion**

Many strains of *P. aeruginosa* are capable of degrading a variety of organic compounds (Aislabie and Atlas 1988, Chapatwala et al. 1990, Hickey and Focht 1990, Higson and Focht 1990, Al-hadhrami et al. 1995). However, *P. aeruginosa* is also a well documented opportunistic pathogen and is the cause of a number of serious infections such as endocarditis, meningitis,
endophthalmitis, malignant external otitis and pneumonia in susceptible individuals (Pollack 1990). Therefore, its use for bioremediation purposes may be limited.

McDade and Shepard (1979) demonstrated that pre-growth conditions exerted a marked influence on the virulence of an opportunistic pathogen to its host. Therefore, this study was designed to determine the potential virulence of 12 different hydrocarbon-degrading strains of

![Graph showing hemolytic activity ratio for P. aeruginosa strains](image_url)

**Fig. 1.** Ratio of hemolytic activity for *P. aeruginosa* strains pre-grown on glucose and a hydrocarbon. The ratio is expressed as the absorbance of hemoglobin liberated by each strain divided by the absorbance of the control. Each result shown is the average of duplicate tests. The strains pre-grown on hexadecane include: ATCC 9027, ATCC 15524, ATCC 27853, ATCC 21472, ATCC 17423, ATCC 15442, ATCC 31479, NRRL 3198, and UG2. Isolate ATCC 19154 was pre-grown on lanolin, JB2 on 2-chlorobenzoate, and KDC on acetonitrile.
Influence of hydrocarbons on *P. aeruginosa*

*P. aeruginosa* pre-grown on either glucose or a hydrocarbon as a sole carbon source (Table 1). Four virulence factors were tested which included antibiotic sensitivity, hemolytic activity, cell adherence and exopolysaccharide capsule formation.

Glucose as a carbon source was used to represent growth conditions of *P. aeruginosa* found in nature in water, soil, and plants (RömLing *et al.* 1994) at uncontaminated sites.

**Fig. 2.** Ratio of bacterial cells adhered to BGM kidney cells for strains of *P. aeruginosa* pre-grown on glucose and a hydrocarbon. Each result shown is the average of duplicate tests. The strains pre-grown on hexadecane include: ATCC 9027, ATCC 15524, ATCC 27853, ATCC 21472, ATCC 17423, ATCC 15442, ATCC 31479, NRRL 3198, and UG2. Isolate ATCC 19154 was pre-grown on lanolin, JB2 on 2-chlorobenzoate, and KDC on acetonitrile.
Studies show that *P. aeruginosa* is becoming increasingly resistant to a number of structurally unrelated antibiotics (Sanders *et al.* 1984, Quinn *et al.* 1988) hence making it an important life-threatening pathogen. There are several mechanisms conferring antibiotic resistance such as antibiotic-inactivation enzymes and decreased permeability of the outer membrane (Burns 1995). However, the results presented here show that most of the strains were multiple-antibiotic-sensitive and exhibited similar antibiotic-susceptibility results when pre-grown on glucose or on the hydrocarbon (Table 2). Therefore, it appears that pre-growth on the hydrocarbon or on glucose did not induce antibiotic resistance.

Two types of hemolysin may be produced by *P. aeruginosa*. One is a heat-stable glycolipid and the other is a heat-labile phospholipase C (PLC) (Liu 1957). Both of these substances act synergistically to break down lipids and lecithin (Pollack 1990). Red blood cells such as human and sheep erythrocytes can be lysed by PLC (Shortridge *et al.* 1992). Additionally, Berk *et al.* (1987) demonstrated that PLC caused death, dermonecrosis, paralysis and inflammation when injected into mice. Other *in vitro* studies have shown PLC to cause both aggregation of human platelets (Coutinho *et al.* 1988), and to be toxic to human leukocytes (Meyers *et al.* 1992).

The results show that all the test strains produced hemolysin and caused sheep red blood cells to lyse. Some of the strains pre-grown on the hydrocarbon exhibited a slightly higher absorbance ratio (hemolytic activity ratio) than the glucose-grown strains (Fig. 1). However, analysis of variance indicated no significant difference in hemolytic activity expressed between the strains pre-grown on glucose and the hydrocarbon (*p* = 0.899).

The ability of *P. aeruginosa* to adhere to tissue cells was the third virulence factor tested. It has been well documented that adhesion to tissue cells by a pathogen is the first step in colonization of the tissue (McEachran and Irvin 1986). Adherence plays an important role in the pathogenicity of *P. aeruginosa*. For instance, this organism adheres to the upper respiratory tract preceding severe pulmonary infections in cystic fibrosis patients (Johanson *et al.* 1979). In addition, adherence to corneal epithelium surfaces precedes major severe corneal infections (Ramphal *et al.* 1981).

### Table 3. Formation of capsules by *P. aeruginosa* strains grown on glucose or hydrocarbon. Smooth indicates capsule formation. Rough indicates no capsule formation. ND = not determined

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose</th>
<th>Hydrocarbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 17423</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>ATCC 19154</td>
<td>smooth</td>
<td>smooth</td>
</tr>
<tr>
<td>ATCC 15524</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>UG2</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>ATCC 9027</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>ATCC 31479</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>JB2</td>
<td>rough</td>
<td>rough</td>
</tr>
<tr>
<td>ATCC 21472</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>KDC</td>
<td>smooth</td>
<td>smooth</td>
</tr>
<tr>
<td>NRRL 3198</td>
<td>rough</td>
<td>rough</td>
</tr>
<tr>
<td>ATCC 27853</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>ATCC 15442</td>
<td>rough</td>
<td>smooth</td>
</tr>
<tr>
<td>CF</td>
<td>smooth</td>
<td>ND</td>
</tr>
<tr>
<td>QC</td>
<td>rough</td>
<td>ND</td>
</tr>
</tbody>
</table>

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The results presented here demonstrate that all the test strains exhibited some degree of adherence to BGM kidney cells (Fig. 2). However, the strains pre-grown on glucose adhered in significantly greater numbers than the strains pre-grown on the hydrocarbon \((p = 0.018)\). Therefore, the test strains may have used different adhesion mechanisms when pre-grown on glucose or the hydrocarbon. Studies have shown that nonmucoid isolates of \(P. aeruginosa\) use pili as adhesins (Woods et al. 1980, Ramphal et al. 1984), while mucoid isolates use exopolysaccharide capsules for adherence (Ramphal and Pier 1985, Doig et al. 1987). Woods et al. (1980) demonstrated that nonmucoid isolates of \(P. aeruginosa\) used pili to adhere to buccal cells of cystic fibrosis patients. They also showed that mucoid isolates of \(P. aeruginosa\) did not adhere as well as non-mucoid isolates to the buccal cells. In addition, Pier et al. (1992) showed that mutant strains of \(P. aeruginosa\) that did not produce pili displayed low levels of colonization of the GI tract of mice. In contrast, other studies have reported that nonmucoid forms do not adhere as well to tissue cells as mucoid forms of \(P. aeruginosa\) (Marcus and Baker 1985, Zanetti et al. 1994).

Capsular production was judged as positive or negative-based colonial appearance. Strains of \(P. aeruginosa\) that produce exopolysaccharide capsules form smooth colonies, while strains that do not produce these capsules form rough colonies. In addition to serving as an adherence factor, exopolysaccharide capsules confer antiphagocytic properties enhancing the virulence of the bacterium (Schwarzmann and Boring 1971).

In this study, 3 of 14 strains and 10 of 12 strains pre-grown on glucose and on a hydrocarbon, respectively, formed smooth colonies on glucose, mucoid colonies on the hydrocarbon, but displayed the greatest degree of cellular adherence when pre-grown on the hydrocarbon. Another exception was strain KDC which formed mucoid colonies on both types of media, but displayed the greatest adherence when pre-grown on the hydrocarbon. Therefore, it is possible that bacterial properties other than capsule formation or pili contributed to the adherence properties of these two strains.

Marcus and Baker (1985) investigated the effect of bacterial properties such as protease production and cell surface hydrophobicity on the adherence of mucoid strains of \(P. aeruginosa\). They demonstrated that proteases altered the epithelial cell surface but had no effect on bacterial adherence. They also found that hydrophobic properties of \(P. aeruginosa\) were not observed, hence adherence of mucoid strains did not correlate with cell surface hydrophobicity. A study by Elsheikh et al. (1985) also showed that hydrophobic interactions of \(P. aeruginosa\) were of little importance to cellular adherence. The role of hydrophobicity, pili production, and protease production in the adherence of \(P. aeruginosa\) strains described here remain to be elucidated in future studies.

The results of this study demonstrate that all the strains expressed virulence factors. Although the degree of expression varied from one virulence factor to another, it does not appear that the presence of a degradable hydrocarbon correlates with a change in the overall virulence of \(P. aeruginosa\).

**Conclusions**

The purpose of this study was to investigate the influence of hydrocarbons on the virulence factors and antibiotic sensitivity of \(P. aeruginosa\) when it is used to degrade hydrocarbons. The expression of virulence factors in response to growth on glucose or a hydrocarbon was examined. The results indicated that: (1) there was no difference in hemolytic activity or antibiotic resistance in the presence of a hydrocarbon or glucose; (2) growth on glucose significantly enhanced cell
adherence, (3) growth on a hydrocarbon enhanced capsule formation; and (4), all the strains expressed virulence factors, but overall growth on a hydrocarbon, such as hexadecane, did not enhance the virulence characteristics of \textit{P. aeruginosa}.

References


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