Assimilation of Hydrocarbons by *Pseudomonas* Strains Isolated from Human Clinical Specimens

JOYCELYN DUNCAN AND JOHN ULRICH

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, and Departments of Microbiology and Pathology, University of New Mexico, Albuquerque, New Mexico 87131

Received for publication 21 June 1973

Twenty strains of *Pseudomonas* isolated from human clinical specimens on routine laboratory media, without hydrocarbon enrichment and unselected for their growth on hydrocarbons, were tested for their ability to utilize a series of eight *n*-alkanes and two *1*-alkenes as a sole carbon and energy source for growth. Hydrocarbon assimilation does occur with such isolates relative to the chain length and the degree of saturation of the hydrocarbon. The data presented show that all 16 strains of *Pseudomonas aeruginosa* studied grew readily on dodecane through hexadecane and on 1-hexadecene. In addition, most strains of this species grew on undecane and 1-dodecene after prolonged incubation. There was a long lag period, usually a minimum of 4 days, before onset of growth on any hydrocarbon. In no case did hexane or decane support growth. Two strains each of *P. maltophilia* and *P. stutzeri* were unable to grow on any of the hydrocarbons tested. Hexane in concentrations above 1% (vol/vol) is bactericidal toward the *Pseudomonas* inoculum. It is toxic even to cells utilizing a different hydrocarbon for growth. The addition of 1% hexane to 1% (vol/vol) hexadecane markedly prolonged the lag phase of *P. aeruginosa* utilizing the hexadecane for growth.

The purpose of this investigation was to determine whether strains of *Pseudomonas* species, especially *Pseudomonas aeruginosa*, cultured from human clinical specimens without hydrocarbon enrichment can utilize a series of *n*-alkanes and *1*-alkenes as a sole carbon and energy source for growth.

**MATERIALS AND METHODS**

Twenty strains belonging to the genus *Pseudomonas* were isolated from clinical material and were maintained at room temperature on Difco nutrient agar slants until subjected to the biochemical and hydrocarbon studies. The biochemical identification was determined according to the methods and charts of King (9).

The hydrocarbon assimilation studies were carried out by adding a single hydrocarbon to each flask of basal salts medium inoculated with the individual strain of *Pseudomonas* being tested. Growth of the organism, as measured by the appearance of turbidity, was used for determining assimilation.

The basal medium was a modification of Bushnell and Haas (2) with the following composition: 0.2 g of MgSO₄, 0.02 g of CaCl₂, 1.0 g of KH₂PO₄, 1.0 g of Na₂HPO₄, 1.0 g of NH₄NO₃, 1.0 mg of FeSO₄·7 H₂O, and distilled water to 1 liter (pH 7.0 to 7.2).

This medium was dispensed in 50-ml portions in 125-ml Erlenmeyer flasks and autoclaved. Cotton plugs were used, and aluminum foil covered the top of the flask.

The inoculum for each hydrocarbon test flask was 0.1 ml of a washed cell suspension containing approximately 3.5 × 10⁻¹⁰ organisms per ml in basal medium. Because both strains of *P. maltophilia* studied required added methionine (5, 8, 14), all hydrocarbon assimilation tests for *P. maltophilia* were carried out with the addition of a final concentration of L-methionine of 100 μg/ml of basal medium.

The hydrocarbons tested were *n*-hexane, *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, 1-dodecene, and 1-hexadecene (all 99±% pure, Humphrey Chemical Co., North Haven, Conn.). A 1% (vol/vol) concentration was used for all hydrocarbons except hexane (see Table 3). They were added immediately prior to incubation of the inoculated test flasks.

Appropriate controls included growth in the basal medium with 1% dextrose, but no growth in the basal medium alone. The sterility of the hydrocarbons was confirmed by culture each time they were used.

Incubation was stationary at 37°C. Growth, as measured by visual turbidity (1, 18), was graded 1+ to 4+. The flasks were observed until they became 4+ or for a minimum of 30 days if negative. Colony counts, done to correlate these gradations with the increase in the number of bacteria per milliliter, showed the following correlation: non-turbid or "negative," 10⁻⁶; 1+, 10⁻³; 2+, 8 × 10⁻⁴; 3+, 2 × 10⁻²; and 4+, greater
than $2 \times 10^{-9}$ organisms/ml. All tests were done in triplicate. All turbid flasks were subcultured onto Difco nutrient agar plates to rule out contamination. Negative flasks were subcultured to assure viability of the inoculum.

The method for testing hexane assimilation was modified by using ground-glass stoppers instead of cotton plugs (16). Concentrations of hexane from 1 to 10% (vol/vol) were tested (see Table 3). Hexane assimilation studies were performed on only eight strains of *P. aeruginosa* and one strain of *P. maltophilia*, because it was apparent that hexane assimilation differed from other hydrocarbon assimilation and additional studies were indicated.

Hexane and hexadecane each were adsorbed with charcoal ("Nuchar," Industrial Chemical Sales, Div. of West Virginia Pulp and Paper Co., New York, N.Y.). One percent adsorbed hexane and adsorbed hexadecane were tested individually by using *P. aeruginosa* no. 2 inoculum, according to the foregoing method (see Table 4).

Finally, assimilation studies were done to test the combined effect of 1% hexane and 1% hexadecane, by using *P. aeruginosa* no. 2 inoculum and, in all other respects, by following the foregoing methods. Control tests using 1% (vol/vol) hexane and 1% (vol/vol) hexadecane individually with the same inoculum were performed simultaneously (see Table 5).

**RESULTS**

Biochemical tests indicated that 16 of the 20 strains studied were *P. aeruginosa*, two were *P. stutzeri*, and two were *P. maltophilia*.

Results of the overall growth patterns obtained in hydrocarbon assimilation tests for all *Pseudomonas* strains are presented in Table 1. A definite pattern of growth emerged dependent upon the number of carbon atoms in the hydrocarbon substrate and the species of *Pseudomonas* tested. All 16 strains of *P. aeruginosa* grew readily on dodecane through hexadecane and on 1-hexadecene. Most strains of this species grew on undecane and 1-dodecene after a prolonged incubation period. In no case did hexane or decane support growth.

Neither strain of *P. maltophilia* studied was able to grow on any of the hydrocarbons tested. Likewise, neither of the two strains of *P. stutzeri* could utilize these hydrocarbons.

Table 2 shows the number of days of incubation required by *P. aeruginosa* to attain maximal growth on each hydrocarbon. Both the length of the carbon chain and the degree of saturation of the hydrocarbon influence the length of the lag phase. Tridecane, tetradecane, pentadecane, and hexadecane usually supported good growth of all *P. aeruginosa* strains within 10 days, whereas undecane, dodecane and the alkenes required much longer incubation periods. There is more rapid initiation and rate of growth with the increasing number and saturation of carbon atoms in the substrate.

All control tests gave expected results. From all test flasks exhibiting growth, the inoculated

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**Table 1. Growth patterns of *Pseudomonas* strains obtained in hydrocarbon assimilation studies**

| Strain                  | Alkanes C6 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | Alkenes C12 | C16 |
|-------------------------|------------|-----|-----|-----|-----|-----|-----|-----|-------------|-----|
| *P. aeruginosa* no. 2   | -          | -   | -   | +   | +   | +   | +   | +   | -           | -   |
| *P. aeruginosa* no. 3   | -          | -   | -   | -   | +   | +   | +   | -   | -           | -   |
| *P. aeruginosa* no. 4   | -          | -   | -   | +   | +   | +   | +   | +   | -           | -   |
| *P. aeruginosa* no. 5   | -          | +   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* no. 6   | -          | +   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* no. 7   | -          | +   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* no. 8   | -          | +   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* no. 9   | -          | +   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* TIR 93  | NT         | -   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* TIR 204 | NT         | -   | -   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* TIR 39  | NT         | -   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* UI 10   | NT         | -   | -   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* VAD 1370| NT         | -   | -   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* UI 24   | NT         | -   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* V. Thaelus| NT      | -   | -   | +   | +   | +   | +   | +   | -           | +   |
| *P. aeruginosa* Mayo    | NT         | -   | +   | +   | +   | +   | +   | +   | -           | +   |
| *P. maltophilia* Mayo   | -          | -   | -   | -   | -   | -   | -   | -   | -           | -   |
| *P. maltophilia* SBH    | NT         | -   | -   | -   | -   | -   | -   | -   | -           | -   |
| *P. stutzeri* Mayo      | NT         | -   | -   | -   | -   | -   | -   | -   | -           | -   |
| *P. stutzeri* ATCC no. 11607 | NT     | -   | -   | -   | -   | -   | -   | -   | -           | -   |

*Symbols: + indicates growth of the organism; – indicates no growth after 30 days of incubation; and NT indicates hydrocarbon not tested.*
**Pseudomonas** was recovered upon subculture. From each test flask showing no turbidity at the end of the incubation period, **Pseudomonas** was recovered upon subculture, indicating the organism was viable but unable to reproduce on the hydrocarbon substrate. This was not true in the case of hexane, however; hexane showed a toxicity toward the bacterial cells.

Preliminary experiments had shown that hexane not only failed to support growth of **P. aeruginosa** no. 2, but that the inoculum could not be subcultured at the end of the incubation period from some of the flasks containing hexane. A series of hexane assimilation tests with **P. aeruginosa** no. 2 was done, by using decreasing concentrations of hexane from 10% to 1% (vol/vol). The results are presented in Table 3. Hexane in concentrations from 1 to 10% (vol/vol) failed to support growth of **P. aeruginosa** no. 2 after 30 days of incubation. One-third of all subcultures from these flasks at the end of the incubation period were sterile. Only when the lowest concentration of hexane (1% vol/vol) was used could the **Pseudomonas** inoculum be recovered upon subculture from all three flasks.

**P. aeruginosa** strains no. 3 through no. 9 were tested with 2% (vol/vol) hexane only, and these strains also failed to grow. They showed predominantly sterile subcultures, substantiating the toxicity of hexane. These results are included in Table 3. Except at the lowest concentration of 1% (vol/vol), hexane adversely affects cell viability.

This deleterious effect was not noted with other hydrocarbons tested. In preliminary experiments, 1%, 3%, and 5% (vol/vol) of hexadecane and 1-hexadecene were tested with **P. aeruginosa** no. 2 inoculum. All three concentrations of each of these hydrocarbons supported good growth of the inoculum; no cell toxicity was evident.

The results of studies on charcoal adsorbed hexane and hexadecane are presented in Table 4. No growth occurred with adsorbed hexane. If the hexane contained a toxic impurity, the latter was not removed by charcoal. Table 4 shows in addition that **P. aeruginosa** no. 2 is able to grow equally well on adsorbed and unadsorbed hexadecane. Therefore, if this hydrocarbon contained a growth promoting impurity, it was not removed by charcoal adsorption.

Results of studies on the combined effect of hexane and hexadecane are presented in Table 5. This experiment was performed to determine whether hexane would have an inhibitory effect upon assimilation of other hydrocarbons by **P. aeruginosa**. The addition of 1% hexane markedly prolonged the lag phase of **P. aeruginosa**

| Days required for maximal growth (4+) | No. of strains reaching maximal growth, according to chain length of the hydrocarbon assimilated |
|--------------------------------------|-------------------------------------------------------------------------------------------------|
|                                      | Alkanes                                                                                         | Alkenes                                                                                      |
|                                      | C6  C10  C11  C12  C13  C14  C15  C16                                                     | C12  C16                                                                                     |
| 4-7                                  | 0     0     2     3     3     7     0     2                                                      | 0     2                                                                                      |
| 8-10                                 | 0     2     6     7     8     9     0     2                                                      | 2     7                                                                                      |
| 11-20                                | 3     5     5     5     5     0     2     7                                                      | 6     4                                                                                      |
| 21-30                                | 4     8     2     1     0     0     0     0                                                      | 6     1                                                                                      |
| 31-45                                | 3     1     1     1     0     0     0     0                                                      | 2     0                                                                                      |
| No growth in more than 30 days       | 8*    16    6     0     0     0     0     0                                                      | 0     0                                                                                      |

* Only eight strains were tested.

**Table 3. Results of studies on hexane toxicity**

| Strain                  | Hexane (%) (vol/vol) | Pseudomonas inoculum recovered on subculture* |
|-------------------------|----------------------|----------------------------------------------|
| **P. aeruginosa** no. 2 | 10                   | Positive 2| Sterile 1 |%
|                         | 8                    | Positive 2| Sterile 1 |%
|                         | 6                    | Positive 2| Sterile 1 |%
|                         | 4                    | Positive 2| Sterile 2 |%
|                         | 2                    | Positive 2| Sterile 2 |%
|                         | 1                    | Positive 3| Sterile 0 |%
| **P. aeruginosa** no. 3-9| 2                   | Positive 3| Sterile 18 |%

* There was no visible growth in flasks with 30 days of incubation.
* Results of triplicate flasks.

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**Table 2. Number of days of incubation required to attain maximal growth of Pseudomonas aeruginosa on hydrocarbons**

| Strain                  | Hexane (%) (vol/vol) | Pseudomonas inoculum recovered on subculture* |
|-------------------------|----------------------|----------------------------------------------|
| **P. aeruginosa** no. 2 | 10                   | Positive 2| Sterile 1 |%
|                         | 8                    | Positive 2| Sterile 1 |%
|                         | 6                    | Positive 2| Sterile 1 |%
|                         | 4                    | Positive 2| Sterile 2 |%
|                         | 2                    | Positive 2| Sterile 2 |%
|                         | 1                    | Positive 3| Sterile 0 |%
| **P. aeruginosa** no. 3-9| 2                   | Positive 3| Sterile 18 |%

* There was no visible growth in flasks with 30 days of incubation.
* Results of triplicate flasks.
TABLE 4. Results of assimilation studies on charcoal-adsorbed hexane and hexadecane

| Hydrocarbon          | Growth in flask (days of incubation) 3 | 4 | 8 | 30 |
|----------------------|----------------------------------------|---|---|----|
| Hexane unadsorbed    | -                                      | - | - | -  |
| Hexane adsorbed      | -                                      | - | - | -  |
| Hexadecane unadsorbed| 2+                                     | 3+| 4+|+  |
| Hexadecane adsorbed  | 2+                                     | 3+| 4+|+  |

* Inoculum: *P. aeruginosa* no. 2; hydrocarbon concentration: 1% (vol/vol).

no. 2 utilizing 1% (vol/vol) hexadecane for growth. Initiation of growth on hexadecane was increased from 4 to 23 days by the addition of 1% (vol/vol) hexane to the culture at the time of inoculation. No pregrowth of bacterial cells on the hexadecane was allowed. Incubation time for maximal growth on hexadecane was increased from 6 to 26 days when hexane was added. Therefore, the toxicity of hexane extends even to cells utilizing a different hydrocarbon for growth. Conversely, the presence of hexadecane exerts a protective influence against the deleterious effects of hexane, but does not cancel them completely.

**DISCUSSION**

The results of this research concur with the results of alkane and alkene oxidation obtained by other authors (3, 4, 7, 10-15, 17, 18). *P. aeruginosa* isolated from human clinical specimens required a long lag phase when utilizing hydrocarbons for growth and exhibited a preference for the longer chain liquid alkanes.

The bactericidal effect of hexane reported for *P. aeruginosa* no. 2 is very similar to the toxicity of hexane for *Mycobacterium smegmatis* obtained by Lukins and Foster (11). These authors found that the addition of 1.5% (vol/vol) hexane completely inhibited growth of *M. smegmatis* on propane, whereas 1% (vol/vol) hexane delayed the onset of growth and markedly decreased the growth rate on propane. The growth rate of the organism on propane plus hexane approached that on propane alone only when the concentration of added hexane was reduced to 0.1% (vol/vol) (11).

If the concentration of hexane and decane had been reduced to less than 1% (vol/vol) in the present investigation, it is possible that growth might have occurred with these hydrocarbons also. On the basis of preliminary experiments and previous publications (10, 13), 1% concentration of hydrocarbon was chosen.

The failure of decane to support growth may not be due to inhibitory concentrations, however. Harris (6) proposed that decane is less readily oxidized than other even-numbered alkanes from C8 through C16 because only decane can assume a ringlike configuration in which there are no exposed methyl groups available for initial oxidation.

The failure of the two strains each of *P. maltophilia* and *P. stutzeri* to assimilate any of the hydrocarbons tested concurs with the negative hydrocarbon assimilation results reported by Stanier et al. (14) for these species. Iizuka and Komagata (8) reported that the five strains of *P. maltophilia* they studied did utilize kerosene for growth when first isolated, but rapidly and permanently lost this ability when subcultured to nutrient agar slants. They had isolated their strains by means of kerosene and crude oil enrichment cultures of oil brine. Considering the source of their isolates, this initial ability to grow on kerosene is not surprising.

Previous publications (10, 13, 14) suggest the possible use of hydrocarbon assimilation tests as an aid in species differentiation. Stanier et al. (14) found that *P. aeruginosa* was the only species which grew on n-dodecane and n-hexadecane. Although only two strains each of *P. maltophilia* and *P. stutzeri* were studied in the present investigation, a larger number of hydrocarbons was tested, and the results are in agreement with Stanier et al. (14). The ability to assimilate hydrocarbons appears to vary with the species and may be of potential diagnostic significance. This possibility should be explored further, with a larger number of strains, to determine whether these species differences are consistent and useful.

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