Growth of *Aeromonas hydrophila* at Low Concentrations of Substrates Added to Tap Water

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The ability of an *Aeromonas hydrophila* isolate obtained from filtered river water to grow at low substrate concentrations was studied in batch experiments with tap water supplied with low concentrations of substrates. Growth was assessed by colony count determinations. The isolate only multiplied in the used tap water (2 to 3 mg of dissolved organic carbon per liter) after the addition of a small amount of an assimilable carbon compound. D-Glucose especially caused growth of the organism even at initial concentrations below 10 µg of C per liter. At initial glucose concentrations below the *K* value (12 µg of C per liter), generation times and yield (colony-forming units per milligram of substrate-C) were nonlinear with 1/initial glucose concentrations and initial glucose concentrations, respectively. From these observations, the maintenance coefficient *m* was calculated (*m* = 0.015 mg of glucose per mg [dry wt] per h at 12°C). At initial concentrations below the *K* value of starch (73 µg of C per liter), no growth was observed, but complete use of starch occurred in these situations after the addition of 10 µg of glucose-C per liter. The results of this study show that information of ecological significance may be obtained by very simple batch experiments. Moreover, the isolate studied may be used in growth experiments to assess the maximum concentration of glucose which might be present in water, particularly tap water.

Representatives of the species *Aeromonas hydrophila* have frequently been observed in dairy products (11), wastewater (2, 19), surface water (5, 8, 19), ground water (21), and tap water (2, 12, 22, 25). The occurrence of *A. hydrophila* in surface water may result from pollution with wastewater, in which it multiplies (19), as well as from its predominant presence in the alimentary tract of fishes (23). The ability of the organism to act as a fish pathogen (7) may also be important in this respect.

Von Wolzogen Kühr (28) demonstrated that *Pseudomonas fermentans*, which is identical to *A. hydrophila*, was present in tap water as a result of contamination of this water by the feces of chironomid larvae which were growing in a sand filter. In tap water, aeromonads usually constitute a minor part of the bacterial flora (25). However, the presence of these organisms is undesirable because they interfere with the determination of coliform bacteria, producing false-positive reactions (12). Moreover, *A. hydrophila* is known to be an opportunistic pathogen for humans (10, 18, 20, 26).

The aim of the present investigation was to obtain information about the growth of *A. hydrophila* at low substrate concentrations as part of an investigation on the behavior of different types of bacteria in tap water.

**MATERIALS AND METHODS**

**Pure cultures.** An aerogenic *A. hydrophila* isolate, strain 315, was obtained from the filtrate of a rapid sand filter supplied with river water. The isolation and identification procedures have been described previously (25). Two additional aerogenic *A. hydrophila* isolates, strains 578 and 666, were obtained from two types of tap water prepared from surface water.

**Basal salts solution.** The solution contained, per liter of demineralized water, the following: NH₄Cl, 0.5 g; Na₂HPO₄·12H₂O, 0.5 g; KH₂PO₄, 2.7 mg; K₂HPO₄·3H₂O, 5.3 mg; MgSO₄·7H₂O, 50 mg; CaCl₂·2H₂O, 50 mg; MnSO₄·H₂O, 5 mg; FeSO₄·7H₂O, 3 mg; ZnSO₄·7H₂O, 0.1 mg; CuSO₄·5H₂O, 0.1 mg; CoCl₂·6H₂O, 0.05 mg. The pH was 6.8 after sterilization.

**Preparation of glassware and medium.** The experiments were performed in 1-liter, calibrated, conical, glass-stoppered Pyrex glass flasks. These flasks were cleaned with a 10% solution of K₂Cr₂O₇ in concentrated H₂SO₄, followed by rinsing with hot tap water, a 10% HNO₃ solution, and hot tap water again. Thereafter, they were heated overnight at 250 to 300°C. The pipettes (1 ml) were cleaned in the same way. The cleaned flasks were filled with 600 ml of tap water (deviation usually less than 3.5%). The tap water originated from the municipal Dune Waterworks of The Hague, where it is prepared from dune-infiltrated ground water.

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river water by the addition of powdered activated carbon, followed by rapid and slow sand filtration, respectively. The final water contained 2 to 3 mg of dissolved organic carbon per liter and 8 to 9 mg of NO₃⁻-N per liter and had a pH between 7.1 and 7.5.

The vegetative cells of the bacteria present in the sampled tap water were killed by placing the flasks in a water bath at 80°C for 3 h. After cooling, NH₄Cl and Na₂HPO₄·12H₂O, usually in concentrations of 1.16 mg of N per liter and 83 μg of P per liter, and organic compounds as sources of energy and growth were added from separately heated solutions. The inorganic compounds were supplied to ascertain that only the organic compounds were growth limiting.

Preparation of the inoculum. After counting cell numbers in a Bürker-Türk counting chamber, a turbid suspension of a 24-h slant culture of strain 315 in sterile tap water was inoculated into a 100-ml infusion bottle containing 50 ml of sterile tap water. This suspended culture that initially contained about 5 × 10⁶ colony-forming units (CFU) per ml was stored at 25°C and repeatedly used for inoculating the test flasks. In some experiments strain 315 was pregrown in the basal salts solution containing β-glucose, acetate, starch, or glycerol at an initial concentration of 1 mg of substrate-C per liter. Strains 578 and 666 were only pregrown in the basal salts solution with 1 mg of acetate-C per liter. Cells pregrown on an added carbon source were only inoculated into the test flasks when the stationary phase (usually 2 × 10⁶ to 3 × 10⁷ CFU/ml at 1 mg of substrate-C per liter) had been reached in the precultivation bottle. The initial concentration of cells in the test flasks usually ranged from 50 to 200 CFU/ml.

Determination of growth. After inoculation, the flasks containing 600 ml of tap water and the test compounds were incubated at 15 ± 1°C. The growth curves were obtained by a regular determination of the colony counts (Nt; CFU per milliliter). For this purpose, the spread plate technique was applied by plating 0.05 ml in triplicate from decimal dilutions on predried Lab-Lemco (Oxoid) agar plates. The colonies of the aeromonads were clearly visible after an incubation period of 18 to 20 h at 25°C. The generation time G (in hours) of the colony-forming cells under the experimental conditions was calculated with the equation \[ G = \log 2 \left( \frac{t' - t}{\log N_t - \log N_i} \right) \], where \( t' - t \) = the incubation time (in hours) during which \( N_t \) increased to \( N_i \). These calculations were performed for the period that the growth curve was found linear with time in a half-logarithmic plot. All experiments were performed in duplicate.

RESULTS

Growth of A. hydrophila strain 315 on different compounds at a concentration of 1 mg of substrate-C per liter. β-Glucose, DL-lactate, acetate, l-glutamate, and succinate were tested as substrates for growth (Table 1). The aeromonad grew immediately on 1 mg of glucose-C per liter, whereas with acetate and glutamate, lag periods of 2 and 7 days were observed (Fig. 1). Growth on succinate and lactate was extremely slow; therefore, maximum colony counts (Nmax) were not estimated. In tap water without added substrate no growth but a die-off of cells was observed, indicating that the organic carbon compounds originally present were not suitable substrates for the aeromonad.

Use of glucose at low concentrations. Two series of experiments (A and B) with glucose revealed that growth of the isolate was caused even by the addition of 2.5 μg of glucose-C per liter of tap water (Table 2). The relationship between \( N_{\text{max}} \) and initial glucose concentration (S) was linear when \( S > 10 \mu g \) of glucose-C per liter (Fig. 2). From this linear relationship, a yield of 3.7 × 10⁶ CFU/mg of glucose-C was calculated. During the experiments of series B, the incubation temperature was 12°C, resulting in a prolonged G at \( S = 1 \) mg of glucose-C per liter, as compared with earlier results (Table 1) when the temperature was 15°C.

As the use of glucose may have been influenced by the precultivation carbon source, an experiment on glucose (10 μg of C per liter) was conducted with cells of strain 315 pregrown in the basal salts solution with 1 mg of acetate-C per liter. The organism was found to grow rapidly with a lag phase less than 24 h, suggesting that its glucose uptake is controlled by a constitutive enzyme system with a high substrate affinity.

Two other representatives of A. hydrophila, strains 578 and 666, obtained from tap water

<table>
<thead>
<tr>
<th>Substrate added* (1 mg of C per liter)</th>
<th>G* (h)</th>
<th>( N_{\text{max}}* \times 10^9 ) CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Acetate</td>
<td>12.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Glutamate</td>
<td>12.9</td>
<td>11.5</td>
</tr>
<tr>
<td>Succinate</td>
<td>63.9</td>
<td>82.4</td>
</tr>
<tr>
<td>Lactate</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* All flasks received 0.15 mg of NH₄⁺-N per liter.

Data from duplicate flasks are presented in corresponding sequence.

—*, No data.

Table 1. Growth of A. hydrophila strain 315 at 15°C in the presence of substrates added to tap water.
were tested for their ability to grow at low glucose concentrations. During an incubation period of 11 days in tap water without glucose, $N_t$ of both isolates decreased. The addition of 10 $\mu$g of glucose-C resulted in distinct growth with $N_{max}$ and $G_s$ identical to those found with strain 315 in a similar situation.

**Use of starch at low concentrations.** *A. hydrophila* may produce extracellular enzymes for the hydrolysis of proteins, blood cells, starch, chitin, and lipids. However, information is lacking about the effect of low concentrations of macromolecular compounds. Experiments with strain 315 revealed that added starch only produced growth when $S$ was $\geq 100 \mu$g of starch-C per liter of tap water. Still no increase of $N_t$ was observed at initial concentrations of 10 and 25 $\mu$g of starch-C per liter after an incubation period of 21 days. On day 21, the inoculum in the bottles without growth was raised with glucose-grown cells to about 1,600 CFU/ml. After another 15 days of incubation, no increase of cells was again observed. Then, 10 $\mu$g of glucose-C per liter was added to the flasks, immediately resulting in growth.

The results of this experiment (Table 3) show that a linear relationship existed between the observed $N_{max}$ values and the starch-C concentrations of 100 $\mu$g per liter and above. The obviously complete use of 100 $\mu$g of starch-C per liter reveals that strain 315 is able to use starch at very low concentrations. This conclusion is supported by the $N_{max}$ values of the starch-containing bottles supplied with 10 $\mu$g of glucose, which approximated the sum of the values that could have been expected by the complete use of both starch and glucose.

**Use of acetate and some other com-**
pounds at low concentrations. Strain 315 was able to use acetate-C at concentrations of 100 μg of C per liter or above (Table 4). Maximum growth rate already occurred at 1 mg of acetate-C per liter. The effect on the growth of the isolate was also tested with succinate, L-glutamate, yeast extract, L-arabinose, D-mannitol, D-glucosamine, and gluconate at initial concentrations below 1 mg of substrate-C per liter of tap water. Neither succinate nor glutamate caused growth when added at an initial concentration of 10 μg of C per liter. An additional supply of 10 μg of glucose-C per liter resulted in rapid growth, but the N_max values indicated that only glucose was used for growth.

Strain 315 also did not multiply with yeast extract, D-glucosamine, and gluconate at concentrations of 10 μg of substrate-C per liter. Growth occurred in the presence of glycerol-C at a concentration of 1 mg/liter, but not at concentrations of 10 and 20 μg/liter. On 10 μg of mannitol-C per liter, no growth was observed within 14 days, but slow and immediate multiplication (G = 68 h) occurred with 20 μg. In an experiment with 10 μg, 20 μg, and 1 mg of arabinose-C per liter with cells from a flask with 20 μg of mannitol-C per liter in which N_max had been reached, growth was observed after a lag period of about 24 h. The Gs were 6.1 h at 1 mg of substrate-C per liter, 27.3 h at 20 μg of substrate-C per liter, and 36.4 h at 10 μg of substrate-C per liter. The results of these experiments indicate that only some specific carbohydrates could produce growth of the aeromonad at initial substrate concentrations below 100 μg of C per liter.

**DISCUSSION**

In this study, N_i was used to determine the G as well as to quantify the amount of biomass. From the linear relationship observed between S and N_max when S was ≥10 μg of glucose-C per liter and on starch and acetate when S was ≥100 μg of substrate-C per liter, it may be concluded that the use of N_i to quantify biomass and to estimate G in the described experiments is justified. With low numbers of cells (<100 CFU/ml) the applied method is not accurate, but this was not important in these cases.

The Gs of A. hydrophila strain 315 observed at different concentrations of a number of substrates may be used to calculate the substrate

![Graph](image)

**Fig. 2.** The observed N_max in relation to the amount of glucose added to tap water. Symbols:
- O—O, series A;
- □—□, series B.

**Table 3. Growth of A. hydrophila strain 315 at 15°C on starch added to tap water**

<table>
<thead>
<tr>
<th>Substrate added (per liter)</th>
<th>Glucose</th>
<th>(N_{max}^*) (CFU/ml)</th>
<th>(G^*) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>10 μg</td>
<td>None</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>10 μg</td>
<td>10 μg</td>
<td>12.4</td>
<td>6.1 × 10^4</td>
</tr>
<tr>
<td>25 μg</td>
<td>None</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>25 μg</td>
<td>10 μg</td>
<td>6.4</td>
<td>1.4 × 10^5</td>
</tr>
<tr>
<td>100 μg</td>
<td>None</td>
<td>3.3</td>
<td>3.0 × 10^5</td>
</tr>
<tr>
<td>1 mg</td>
<td>None</td>
<td>3.3</td>
<td>3.2 × 10^7</td>
</tr>
</tbody>
</table>

* Inoculum derived from a flask from series A initially containing 100 μg of glucose-C per liter.

* Glucose was added after an incubation period of 36 days.

* Data from duplicate flasks are presented in corresponding sequence.
Acetate added (mg of C per liter) | G⁺ (h) | \( N_{\text{max}} \) (CFU/ml)
---|---|---
None | No growth | No growth | No growth | No growth | No growth
0.025 | 0.1 | 1.0 | 10.0 | 0.1 | 21.7 | 21.9 | 3.5 \( \times \) 10⁶
| 21.9 | 3.5 \( \times \) 10⁶ | 16.5 | 3.0 \( \times \) 10⁶
| 16.5 | 2.9 \( \times \) 10⁷ | 14.8 | 2.9 \( \times \) 10⁷

* Inoculum from stored infusion bottle.
* Data from duplicate flasks are presented in corresponding sequence.
* — , No data.

concentration \( (K_s) \), which produces half the maximum growth rate. For this purpose a slightly adapted Lineweaver-Burk modification of the Monod equation was used:

\[
G = \frac{G_{\text{min}}}{1 + (G_{\text{min}})(K_s)(1/S)}
\]

in which \( G \) is given in hours at substrate concentration \( S \), and \( G_{\text{min}} = \text{minimal} \ G \). \( K_s \) is the substrate concentration at which \( G = 2 \times G_{\text{min}} \).

The relationship between \( G \) and the reciprocal value of the \( S \) (Fig. 3) is linear at \( S \) above 10 µg/liter. Calculation of this function produced the equation:

\[
G = 7.17 + (0.086)(1/S)
\]

According to equation (2), the \( K_s \) value of strain 315 equals 12 µg of glucose-C per liter. Similar calculations with the results of the experiments with starch, acetate, and arabinose gave \( K_s \) values of 73, 40, and 38 µg of C per liter, respectively.

At \( S \) below 10 µg of C per liter, nonlinear relationships were observed between \( N_{\text{max}} \) and \( S \) (Fig. 2) and between \( G \) and \( 1/S \) (Fig. 3). The differences between the observed \( G_s \) \( (G_{\text{obs}}) \) and those following from equation 2 \( (G_{\text{calc}}) \) may be expressed by the equation:

\[
t^-1 = G_{\text{calc}} - G_{\text{obs}}
\]

in which \( t^- \) is the mean half-life assigned to endogenous metabolism. When \( S = 2.5 \) µg of glucose-C per liter, \( G_{\text{calc}} = 41.4 \) h and \( t^- = 95.7 \) h. When \( S = 5 \) µg of glucose-C per liter, \( G_{\text{calc}} = 24.3 \) h and \( t^- = 93.5 \) h. Obviously, the \( t^- \) values were similar at the two glucose concentrations, and strain 315 had a half-life of nearly 4 days in these situations.

From the \( t^- \), the specific endogenous metabolism rate \( \mu_e \) may be calculated with \( \mu_e = 0.693/t^- \). This produces \( \mu_e = 7.33 \times 10^{-3} \) h⁻¹. According to Pirt (17), the maintenance coefficient \( m \) (milligrams of substrate per milligram [dry weight] per h) can be calculated from \( m = \mu_e \times Y_G \) \( (Y_G = \text{yield corrected for endogenous use of substrate, grams [dry weight] per gram of substrate}) \). Although \( Y_G \) cannot be estimated directly, it will not differ much from yields measured in batch cultures at high initial substrate concentrations. Previous experiments revealed that these yields varied between 0.43 to 0.53 g (dry wt)/g of glucose for some fluorescent pseudomonads and an aeromonad. The \( m \) of strain 315 therefore approximates 0.015 mg of glucose per mg (dry wt) per h. This value is lower than those reported by Pirt (17) for Aerobacter aerogenes \( (m = 0.076 \) on glycerol at 37°C) and Aerobacter cloacae \( (m = 0.094 \) on glucose at 37°C) and those reported by Palumbo and Witter (16) for Pseudomonas fluorescens \( (m = 0.20 \) on glucose at 20°C). The difference with the values reported by Pirt (17) might be explained by the low temperature (12°C instead of 37°C) at which strain 315 was grown on glucose.

The experiments with glucose, arabinose, and mannitol revealed that strain 315 multiplied on these compounds even at initial substrate concentrations below \( K_s \). On acetate and on starch no growth occurred at corresponding concentrations. However, the observations presented in Tables 3 and 4 indicate that these compounds could be used at low concentrations once growth had started. Enzyme production probably was not induced in nongrowing cells at starch–C concentrations below the \( K_s \) value but occurred at such concentrations when the cells were multiplying as a result of added glucose.

The determined \( K_s \) value of strain 315 on glucose is very low as compared with some growth and transport constants of other bacteria on this substrate, e.g., Pseudomonas aeruginosa, 0.5 to 0.8 mg of glucose-C per liter (3, 14); P. fluorescens, 72 µg of glucose-C per liter (13); and Escherichia coli, 72 µg of glucose-C per liter (27). This suggests that the aeromonad may compete effectively with these bacteria if glucose would be growth limiting. As both drinking water isolates and strain 315, which was of surface-water origin, multiplied at very low glucose concentrations, it is suggested that this property may be rather common among representatives of the species A. hydrophila. The minor contribution of the aeromonads to the bacterial populations of drinking water (22, 25) and surface water (5, 19), in which glucose

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**Table 4. Growth of A. hydrophila strain 315 at 12°C on acetate added to tap water**

<table>
<thead>
<tr>
<th>Acetate added (mg of C per liter)</th>
<th>G⁺ (h)</th>
<th>( N_{\text{max}} ) (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>0.025</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>21.7</td>
<td>21.9</td>
<td>3.5 ( \times ) 10⁶</td>
</tr>
<tr>
<td>15.5</td>
<td>15.8</td>
<td>3.0 ( \times ) 10⁶</td>
</tr>
<tr>
<td>16.5</td>
<td>14.8</td>
<td>2.9 ( \times ) 10⁷</td>
</tr>
<tr>
<td>16.5</td>
<td>2.9 ( \times ) 10⁷</td>
<td></td>
</tr>
</tbody>
</table>

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The determined \( K_s \) value of strain 315 on glucose is very low as compared with some growth and transport constants of other bacteria on this substrate, e.g., Pseudomonas aeruginosa, 0.5 to 0.8 mg of glucose-C per liter (3, 14); P. fluorescens, 72 µg of glucose-C per liter (13); and Escherichia coli, 72 µg of glucose-C per liter (27). This suggests that the aeromonad may compete effectively with these bacteria if glucose would be growth limiting. As both drinking water isolates and strain 315, which was of surface-water origin, multiplied at very low glucose concentrations, it is suggested that this property may be rather common among representatives of the species A. hydrophila. The minor contribution of the aeromonads to the bacterial populations of drinking water (22, 25) and surface water (5, 19), in which glucose
concentrations are in the range of some micrograms per liter (29), may be explained by the presence of many other bacteria having glucose transport systems with higher affinity than the aeromonads (9, 15, 24) and the inability of the aeromonad studied to multiply at low concentrations of acetate, lactate, succinate, and glutamate.

The organic carbon compounds originally present in the used tap water (dissolved organic carbon = 2 to 3 mg/liter) were not used as sources for carbon and energy of the A. hydrophila strains tested. With equation 2 the maximum glucose concentration at which strain 315 does not grow can be calculated. In this situation $G_{\text{calc}}$ equals $t_{\text{m}}$, which is 94.6 h, and $S = 0.98 \mu g$ of glucose-C per liter. Strain 315 was unable to maintain itself in the tap water used; therefore, the glucose concentration in this water was always below 1 $\mu g$ of C per liter. This calculation demonstrates that growth experiments as described may be used to measure the concentration of substrate available for a test strain in drinking water or in water from different treatment stages to assess treatment efficiency regarding substrate removal. For this purpose, representatives of specific species, particularly P. aeruginosa, which multiplies in clean water without a carbon source added (1, 4), may be selected. With the $N_{\text{max}}$ values the concentration of assimilable compounds originally present may be expressed in equivalents of a substrate on which the yield (CFU per milligram of substrate-C) is known. In addition, $G_{\text{s}}$ may be used to calculate the maximum concentration of certain substrates that might be present in water. For such calculations $G_{\text{min}}$ and $K_{\text{s}}$ values should be known.

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LITERATURE CITED


