Development of an Oxygen-resistant Hydrogen Bacterium during Plate Cultivation under High Oxygen Tension

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An oxygen-resistant hydrogen bacterium developed during an autotrophic plate culture under 40% O2. When oxygen-sensitive strain N34 was incubated under an atmosphere of 50% H2 + 40% O2 + 10% CO2, small white colonies (SW) first formed, and then, large yellow colonies (LY) developed from some of the SW. The LY counts increased gradually. The hydrogenase activity of SW was one-fifth that of LY. The growth lag period of a liquid culture under 40% O2 inoculated with SW was more than 100 hr, while it was almost zero with LY, indicating that SW were oxygen-sensitive and LY oxygen-resistant. When a single SW was transferred to a fresh plate culture under 40% O2 at first SW and then LY were formed again. On the other hand, LY were always formed under 40% O2 when a single LY was transferred. From these data, it was concluded that SW were formed from oxygen-sensitive strain N34 whose growth was inhibited under 40% O2 and that oxygen-resistant LY were formed as a result of adaptation of SW to high oxygen tension.

The formation of two types of colonies has been reported for several microorganisms as follows.

Colony dimorphism in Derxia gummosa was first described as a few isolated “massive” colonies among many “thin” whitish colonies. When a thin colony as well as a massive colony was reinoculated, each of them regularly showed the same colony dimorphism.1) The influence of oxygen concentration on the colony type of Derxia gummosa grown on nitrogen-free media was reported.2) At an oxygen concentration of less than 20% D. gummosa formed only the “massive” colony type; the “thin” colony type that was predominant in air was almost completely absent. Only the “massive” colonies reduced acetylene. The inhibitory effect of oxygen on growth in nitrogen-free media was shown to influence the colony morphology.2)

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Similar colony dimorphism was also reported in a mutant of Azotobacter vinelandii.3) On the other hand, dissociation of Pseudomonas aeruginosa was investigated in the medical field to establish a typing method for the bacteria.4) In that case, a large, flat and moist colony (designated as type la) and a small, round and convex colony (designated as type sm) were isolated on an agar plate. The former developed from the latter after incubation for several days.

We have already reported the development of an oxygen-resistant hydrogen bacterium during a liquid cultivation inoculated with oxygen-sensitive strain N34 under an atmosphere of 50% H2 + 40% O2 + 10% CO2.5) To explain this phenomenon there were two hypotheses; the adaptation of oxygen-sensitive cells to 40% O2 and the selection of oxygen-resistant variants that might have preexisted in the inoculum. In the previous paper,5) we speculated that the former was possible. After that, we tried to substantiate this speculation with other evidence. And then, we observed the formation of two types of colonies on an agar plate which was inoculated with oxygen-sensitive strain N34 and incubated under an atmosphere of 50% H2 + 40% O2 + 10% CO2.

In this paper we report the formation of two types of colonies and the differences between
the two types. And evidence of the development of an oxygen-resistant hydrogen bacterium from oxygen-sensitive strain N34 is presented here.

**MATERIALS AND METHODS**

**Organism.** A hydrogen bacterium strain, N34,6) isolated from oily soil in the northern district of Japan was used.

**Medium.** The medium used for chemoautotrophic growth was composed of 300 mg KH₂PO₄, 400 mg K₂HPO₄, 1 g urea, 200 mg MgSO₄·7H₂O, 50 mg FeSO₄·7H₂O, 0.05 mg ZnSO₄·7H₂O, and 0.1 mg Na₂MoO₄·2H₂O, made up to 1 liter with distilled water. The urea solution was filter-sterilized (membrane filter; pore size, 0.22 μm) separately and added aseptically.

**Incubation of plates.** Standard 9 cm diameter petri dishes were used throughout the work. Cells were diluted to about 10⁵/ml with 0.022 M K-phosphate buffer (pH 7.0) and inoculated on agar plates with a Spiral Plate Maker (Spiral Systems Marketing, model O). Incubation of the plates was carried out in a desiccator under an atmosphere of 50% H₂+40% O₂+10% CO₂ or 80% H₂+10% O₂+10% CO₂ at 35°C.

**Colony counting procedures.** Colony counts were determined by direct visual inspection with a Colony Viewer (Spiral Systems Marketing, model MV). Total viable counts were calculated from the colony counts.

**Extraction and analysis of pigment.** Colonies were collected in 0.022 M K-phosphate buffer (pH 7.0) and centrifuged at 10,000 x g for 15 min. Wet cells were extracted with acetone-methanol (3:1, v/v) at room temperature until they became colorless. Absorption spectra were measured with a spectrophotometer (Shimadzu, model UV-2000S).

**Measurement of growth lag period.** Liquid cultures were carried out in 1-liter suction flasks containing 10 ml of media under an atmosphere of 50% H₂+40% O₂+10% CO₂ at 35°C. Cell growth was determined by measuring the optical density with a photometer (Shimadzu, model Spectronic 20) at 450 nm. The growth lag period was determined as the period between the start of the culture and the measuring time just before the apparent increase in O.D.

**Measurement of hydrogenase activity.** The hydrogenase activity was measured manometrically according to the method described by Aggag et al.7) using methylene blue as electron acceptor. The activity was expressed as the uptake rate of H₂ (μl/hr/mg protein).

**Protein determination.** Protein content of a cell suspension was determined by the Lowry method8) using bovine serum albumin as protein standard.

**RESULTS**

**Formation of two types of colonies under 40% O₂**

Two types of colonies were formed during a plate culture under 40% O₂ which was inoculated with an oxygen-sensitive hydrogen bacterium strain N34.6) A single colony of strain N34 previously grown on an agar plate under an atmosphere of 80% H₂+10% O₂+10% CO₂ was suspended in 3 ml of 0.022 M K-phosphate buffer (pH 7.0), diluted 10²-fold and spread on agar plates. After incubation in a desiccator under an atmosphere of 50% H₂+40% O₂+10% CO₂ at 35°C for 10 days two types of colonies, small white colonies and large yellow colonies, were formed on an agar plate as shown in Fig. 1. The former was designated as SW and the latter as LY.

When the colony formation was observed daily, SW and LY appeared after 2 to 3 days and 5 to 6 days incubation, respectively. LY did not develop on a vacant space of the plate but from some of the SW. This result was substantiated by the observation of the de-
TABLE I. INCREASE OF LARGE YELLOW COLONIES (LY) DURING INCUBATION UNDER 40% O$_2$

The plate culture was carried out in a desiccator under an atmosphere of 50% H$_2$+40% O$_2$+10% CO$_2$ at 35°C.

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Colony counts on plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>167</td>
</tr>
<tr>
<td>10</td>
<td>352</td>
</tr>
<tr>
<td>15</td>
<td>474</td>
</tr>
</tbody>
</table>

Fig. 2. Photomicrographs of Cells of SW and LY Grown under 40% O$_2$ for 10 Days.

Development of LY from SW which were marked with points on the bottom of the petri dish. LY developed not only from widely separated SW but also within the area of high cell density. The LY counts increased gradually as shown in Table I, and as a result, various sizes of LY were found on the plate as shown in Fig. 1.

Fig. 3. Absorption Spectra of Acetone–Methanol Extracts from Washed Cells of LY (——) and SW (—–—–).

Difference between the two types of colonies

Differences between the two types of colonies, SW and LY, were investigated; they were cell morphology, pigment formation, hydrogenase activity, oxygen-sensitivity and colony type when transferred to fresh medium.

Figure 2 shows photomicrographs of cells of SW and LY grown under 40% O$_2$ for 10 days. No major difference was found in the cell morphology of the two types of colonies.

The pigment of LY was extracted with acetone–methanol (3:1, v/v) and the absorption spectrum was measured. Absorption peaks were found at 428, 453 and 480 nm (Fig. 3). These absorption peaks indicated that the pigment of LY was zeaxanthin dirhamnoside,$^{10}$ a kind of carotenoid, which had been isolated from *Xanthobacter autotrophicus* strain 7C.$^{11}$ The same absorption peaks were also found in extracts from SW of high cell
density (Fig. 3).

Hydrogenase activities (μl H₂/hr/mg protein) of SW and LY were 74.3 and 342.9, respectively; the activity of SW was about one-fifth that of LY.

The oxygen-sensitivity of SW and LY was examined by measuring the growth lag period in liquid cultures under 40% O₂ inoculated with each of SW and LY. The growth lag period with SW was 138 hr, while it was almost zero with LY, indicating that SW was oxygen-sensitive and LY was oxygen-resistant.

The colony formation was compared between SW and LY when transferred to fresh plate cultures under 40% O₂ and 10% O₂. The results are summarized in Fig. 4. When a single colony of SW was transferred to a fresh plate culture under 40% O₂, at first SW and later LY were formed again, while only LY were formed when a single colony of LY was transferred. On the other hand, when each of SW and LY was transferred to a fresh plate culture under 10% O₂, LY were always formed in each case. But LY which were formed under 10% O₂ from SW as the inoculum were oxygen-sensitive (OS). These oxygen-sensitive LY were also formed when oxygen-sensitive strain N34 was usually cultivated under 10% O₂ as seed cultures. In order to distinguish them from oxygen-resistant (OR) LY, these oxygen-sensitive apparent LY are designated as ‘LY’ in Fig. 4. In these colony formations, only the development of LY from SW under 40% O₂ was irreversible.

From these results, it was concluded that SW were formed from oxygen-sensitive hydrogen bacterium strain N34 whose growth was inhibited by 40% O₂ and that LY were formed as a result of adaptation of strain N34 to 40% O₂.

**Increase of oxygen-resistant cells during 40% O₂-culture**

The time course of viable counts in 40% O₂-liquid cultures inoculated with oxygen-sensitive strain N34 was investigated by calculating the colony counts of SW and LY. The 40% O₂-liquid cultures were started using sixteen flasks. The broth of two flasks was sampled at each indicated time in Table II, diluted appropriately and spread on agar plates.

**Table II. Increase of Oxygen-resistant Cells in 40% O₂-Liquid Cultures Inoculated with Oxygen-sensitive Strain N34**

<table>
<thead>
<tr>
<th>Culture time (hr)</th>
<th>O.D. at 450 nm</th>
<th>O₂-Sensitive cells/ml</th>
<th>O₂-Resistant cells/ml (A)</th>
<th>Total cells/ml (B)</th>
<th>A/B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.187</td>
<td>1.07 × 10⁸</td>
<td>0</td>
<td>1.07 × 10⁸</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.225</td>
<td>1.97 × 10⁸</td>
<td>5.40 × 10</td>
<td>1.97 × 10⁸</td>
<td>2.7 × 10⁻⁵</td>
</tr>
<tr>
<td>47</td>
<td>0.220</td>
<td>2.31 × 10⁸</td>
<td>5.05 × 10</td>
<td>2.31 × 10⁸</td>
<td>2.2 × 10⁻³</td>
</tr>
<tr>
<td>72</td>
<td>0.211</td>
<td>2.10 × 10⁸</td>
<td>4.11 × 10</td>
<td>2.10 × 10⁸</td>
<td>0.2</td>
</tr>
<tr>
<td>94</td>
<td>0.218</td>
<td>1.89 × 10⁸</td>
<td>2.24 × 10</td>
<td>2.11 × 10⁸</td>
<td>10.6</td>
</tr>
<tr>
<td>105</td>
<td>0.482</td>
<td>1.82 × 10⁸</td>
<td>2.55 × 10</td>
<td>4.37 × 10⁸</td>
<td>58.4</td>
</tr>
<tr>
<td>118</td>
<td>1.084</td>
<td>1.81 × 10⁸</td>
<td>1.85 × 10</td>
<td>2.03 × 10⁸</td>
<td>91.1</td>
</tr>
<tr>
<td>126</td>
<td>1.495</td>
<td>1.68 × 10⁸</td>
<td>3.22 × 10</td>
<td>3.39 × 10⁸</td>
<td>95.0</td>
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</tbody>
</table>
plates were incubated under an atmosphere of 50% H₂ + 40% O₂ + 10% CO₂ at 35°C. At first, counts of SW were determined after 2 to 3 days incubation and then after 4 to 5 days counts of LY of uniform size which had been formed simultaneously were determined. Because the LY of uniform size were much larger than LY which developed from SW, the former were clearly distinguishable from the latter. The viable counts of oxygen-sensitive cells and oxygen-resistant cells in the broth were calculated from the colony counts of SW and LY, respectively.

The results are summarized in Table II, in which the data are the means of two samples. It was shown that oxygen-resistant cells developed gradually during the growth lag phase and an increase in O.D. was observed when the viable counts of the oxygen-resistant cells reached 10⁷ cells/ml. Finally, oxygen-resistant cells may account for almost 100 percent of cells in the broth.

**DISCUSSION**

The formation of two types of colonies depending on oxygen tension was reported for *Derxia gummosa* in which “massive” colonies probably occurred when the local oxygen concentration on the agar surface was depressed. In that case, the “massive” colonies were not oxygen-resistant, so the factor controlling the colony morphology appeared to be physiological rather than genetic. In contrast, the development of oxygen-resistant LY from oxygen-sensitive SW under 40% O₂ was irreversible (Fig. 4). The formation of two types of colonies in this work does not resemble the colony dimorphism reported for *D. gummosa* but the dissociation reported *Pseudomonas aeruginosa* with respect to the genetic change of cell morphology.

The question of whether the development of oxygen-resistant LY resulted from adaptation of SW to high oxygen tension or from selection of oxygen-resistant variants that might have preexisted in the inoculum of strain N34 was answered by the following considerations. If it is presumed that the inoculum consisted of a majority of oxygen-sensitive cells and a minority of oxygen-resistant variants and that SW and LY were formed from the former and the latter, respectively, one may expect that LY should be formed simultaneously with a uniform size as observed when the diluted broth from a 40% O₂-culture of strain N34 was inoculated, and that SW should produce only SW when transferred to a fresh plate culture under 40% O₂. But actually, LY were formed gradually with various sizes and SW always produced two types of colonies, SW and LY, when transferred to a fresh plate culture under 40% O₂. These results were sufficient to rule out the latter hypothesis; the preexistence of oxygen-resistant variants in the inoculum of strain N34. In addition, the development of LY from SW was clearly observed by marking widely separated SW with points on the bottom of the petri dish. From these results, it is evident that oxygen-resistant LY developed from SW by adaptation of oxygen-sensitive strain N34 to high oxygen tension.

Carotenoids present in photosynthetic organisms have been reported to have the function of protection against photodynamic killing by oxygen and light. Moreover, the protective effect of carotenoids has been demonstrated in several non-photosynthetic bacteria such as *Corynebacterium poinsettiae* and *Sarcina lutea*. Recently, it was reported that carotenoids appeared to protect the nitrogenase of *Azospirillum brasilense* from oxidative damage. In the case of strain N34, however, carotenoids were produced in SW as well as LY (Fig. 3), indicating that carotenoids were not related to the development of LY from SW under 40% O₂. In addition, since oxygen-sensitive ‘LY’ were formed under 10% O₂ from SW as inoculum (Fig. 4), carotenoids appeared not to have a protective function against oxidative damage.

In the previous paper, we reported the development of an oxygen-resistant hydrogen bacterium during a liquid cultivation which was inoculated with oxygen-sensitive strain.
N34 under an atmosphere of 50% H₂ + 40% O₂ + 10% CO₂. We also reported that the hydrogenase synthesis was repressed markedly by 40% O₂ in oxygen-sensitive strain N34, while it was clearly exhibited in the oxygen-resistant strain N34.16) Probably SW and LY correspond to oxygen-sensitive strain N34 and oxygen-resistant N34, respectively. The difference of hydrogenase synthesis under 40% O₂ between SW and LY may account for the difference of hydrogenase activity between the two types of colonies.

That oxygen-resistant cells increased gradually during the growth lag phase in a 40% O₂-culture which had been shown by the indirect observation in the previous study,5) was substantiated by the calculation of viable counts of oxygen-resistant cells (Table II).

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REFERENCES