We screened soil samples for CO₂-requiring extreme oligotrophs similar to *Rhodococcus erythropolis* N9T-4, which can grow on a basal salt agar medium without an organic carbon source. From 387 soil samples, three isolates were obtained and identified as *Streptomyces* spp. by 16S rDNA analysis. The isolates required gaseous CO₂ for growth and grew on a basal salt medium solidified by silica gel. These results suggest that such CO₂-requiring oligotrophs occur widely in nature.

**Key words:** carbon dioxide-requiring bacteria; oligotroph; *Streptomyces*; silica gel plate

Oligotrophic bacteria have been defined as those that develop at the first cultivation from nature on media with minimal organic matter contents, about 1–15 mg of carbon per liter.¹ Many researchers have focused on their growth at extremely low concentrations of the carbon source and have isolated many oligotrophs from various natural environments, but biochemical and genetic studies are lacking. Although it is thought that oligotrophs scavenge trace amounts of nutrients from the atmosphere, little attention has been given to date to the CO₂ requirement for oligotrophs, but it is possible that some oligotrophs fix CO₂ using low concentrations of other carbon sources. In a previous study, we isolated an extremely oligotrophic bacterium, *Rhodococcus erythropolis* N9T-4, from crude oil stored in a Japanese oil stockpile.² This bacterium grew on a minimal salt medium solidified with agar or silica gel without any additional carbon or energy sources. Strain N9T-4 did not grow under CO₂-limiting conditions, but grew on a NaHCO₃-containing medium under the same conditions, suggesting that the oligotrophic growth of N9T-4 depends on CO₂. Furthermore, we found that other type cultures of *R. erythropolis* showed the same oligotrophic growth as that of N9T-4.² It should be noted that we do not use the term “autotrophic” but rather “oligotrophic” for the growth of N9T-4, since N9T-4 cells have no detectable key enzyme activities in the four microbial CO₂ fixation systems known thus far.³–⁶ Although the CO₂ fixation system of this bacterium remains unknown, oligotrophic conditions induced the enzyme activities involved in formaldehyde oxidation and assimilation, suggesting a metabolic flow from CO₂ to formaldehyde.² It is important to determine whether the characteristics of this type of oligotroph are due to specific environmental conditions such as crude oil,³ a common feature in *Rhodococcus* sp., or are widely found in microorganisms in nature. For this reason, in this study, we screened soil samples for microorganisms that show growth similar to *R. erythropolis* N9T-4.

A total of 387 soil samples were collected from various places: at the seaside, on the roadside, in fields of rice, in a pile of fallen leaves, and so on. The procedure of the screening was divided into three steps, as follows: In the first step, a spoonful of a soil sample was added to 5 ml of 0.85% KCl and mixed well. After the mixture stood for a few minutes, the supernatant was streaked onto a plate consisting of a basal salt medium (BSM), and incubated aerobically at 30°C. From the plates on which microbial growth was observed in several days, 178 bacteria were isolated. In the second step, the 178 isolates were incubated under atmospheric CO₂ and CO₂-limiting conditions. Fourteen isolates showed little or no growth under the CO₂-limiting conditions (Fig. 1). There was no difference in the growth of the other 164 isolates under atmospheric CO₂ and CO₂-limiting conditions, suggesting that these isolates utilized agar (see no. 95-2 in Fig. 1). In the third step, these 14 isolates were incubated under CO₂-limiting conditions on BSM containing 1.5% NaHCO₃ or KHCO₃. We have found that *R. erythropolis* N9T-4 grew under CO₂-limiting conditions on NaHCO₃-containing medium, but not on KHCO₃-containing medium. By contrast, none of the isolates in this study grew under the above conditions (Table 1). Hence a BSM plate inoculated with each isolate was incubated at 30°C for several days in a pouch in which CO₂ gas was generated. Four of the 14 isolates showed good growth under CO₂-limiting conditions, and were identified as *Streptomyces* sp. using 16S rDNA analysis. These results suggest that such CO₂-requiring oligotrophs occur widely in nature.

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growth under the CO$_2$-limiting conditions (Table 1). These results suggest that these isolates used gaseous CO$_2$ directly for oligotrophic growth and that they do not have a carbonic anhydrase that converts HCO$_3^-$ to CO$_2$ and H$_2$O.\textsuperscript{8} None of the isolates showed growth in liquid BSM, which is consistent with the fact that they required gaseous CO$_2$. But we cannot exclude the possibility that the isolates assimilated minor components in agar, which were replaced with hydroxyl groups in the sugar chains of agar,\textsuperscript{9} although no liquidation of agar with the growth of the isolates was observed on the BSM plates. Hence we used a strictly inorganic BSM medium solidified by silica gel\textsuperscript{10} to verify the extremely oligotrophic growth of the isolates. The isolates, except for isolate no. 59, grew on the BSM plates solidified by silica gel, and diffusion of the colonies was observed (Fig. 2). Since complete drying of the silica gel plates caused cracks on the surface, it was difficult to dry silica gel plates appropriately; this is probably what caused diffusion of the colonies. Furthermore, none of the isolates grew on the BSM silica gel plates under CO$_2$-limiting conditions (data not shown). These results indicate that the oligotrophic growth of the isolates in this study was not due to assimilation of agar in the cultivation media. To identify three isolates that exhibited oligotrophic growth on the BSM silica plates, their 16S rDNAs were amplified by PCR and sequenced, followed by phylogenetic analysis. The set of primers used for PCR amplification were Uni 530F and Eu/Ar 1,510R, corresponding to the sequences of \textit{E. coli} 16S rDNA at positions 514 to 530 and at positions 1,510 to 1,492 respectively. The nucleotide sequence data obtained in this study have been submitted to DDBJ (accession nos. AB287516 to AB287518).

Table 1. Effect of Bicarbonates and Gaseous CO$_2$ on the Oligotrophic Growth of the Isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>BSM without CO$_2$ absorbents</th>
<th>BSM with CO$_2$ absorbents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>NaHCO$_3$\textsuperscript{a}</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>31</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>56</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>59</td>
<td>+++</td>
<td>—</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The concentration in BSM was 1.5%.

\textsuperscript{b}CO$_2$ gas was produced by mixing 8 ml of 1.45 M H$_2$SO$_4$ and 10 ml of 50 mM NaHCO$_3$ in a petri dish.

![Fig. 1. Oligotrophic Growth of Representative Isolates.](image1)

This figure shows the oligotrophic growth of representative isolates in the second step of screening. These isolates were incubated at 30 °C for 5 d on a BSM plate in a pouch with (lower photographs) or without (upper photographs) CO$_2$ absorbents. For reference, the growth of isolate no. 95-2, which was screened in the first step of screening but excluded in the second step, are also shown. The BSM contained 1 g NaNO$_3$, 1 g K$_2$HPO$_4$, 0.5 g MgSO$_4$•7H$_2$O, 0.1 g CaCl$_2$•2H$_2$O, 15 g purified agar (Nacalai Tesque, Kyoto, Japan), 0.1% (v/v) vitamin mixture, and 1.0% (v/v) metal solutions in 1,000 ml of deionized water (pH 7.0). The composition of the metal solutions was as follows: 2.0 g MnSO$_4$, 2.2 g ZnSO$_4$•7H$_2$O, 0.26 g CuSO$_4$, 0.26 g Na$_2$MoO$_4$•2H$_2$O, 0.4 g H$_3$BO$_3$, and 0.06 g KI in 1000 ml of deionized water. The vitamin mixture had the following composition: 1 mg thiamin•HCl, 2 mg riboflavin, 2 mg Ca-pantothenate, 2 mg pyridoxine•HCl, 0.1 mg biotin, 1 mg p-aminobenzoic acid, 2 mg nicotinic acid, and 0.1 mg folic acid in 100 ml of deionized water. To create CO$_2$-limiting conditions, 30 g of CO$_2$ absorbent granules (SodaSorb, W. R. Grace, Epernon Cedex, France) was added to a Petri dish and placed in a plastic pouch (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo) with inoculated plates.

![Fig. 2. Inorganic Growth of Isolates on BSM Silica Gel Plates.](image2)

Four CO$_2$-requiring isolates confirmed in the third step of screening were cultivated at 30 °C for several days on BSM plates solidified with silica gel. A petri dish containing water was also put into each pouch to prevent drying of the silica gel.
mined that the isolates nos. 3, 31, and 56 were close to *Streptomyces viridobrunneus* (accession no. AB184714, 99% similarity), *S. bikiniensis* (accession no. AB184602, 99% similarity), and *S. exfoliatus* (accession no. AB184324, 99% similarity) respectively. Based on these results, four laboratory-stock strains belonging to the genus *Streptomyces* (*S. aureus* NBRC 100912, *S. griseus* subsp. alpha NBRC 15421, *S. bikiniensis* NBRC 14598, and *S. venezuelae* NBRC 13096) were subjected to the screening procedures described above. In the first step of the procedure, three strains (*S. venezuelae*, *S. griseus*, and *S. aureus*) grew on BSM agar plates. Among these, only *S. venezuelae* exhibited remarkable oligotrophic growth and did not grow under the CO₂-limiting conditions in the second step (data not shown).

In conclusion, the three *Streptomyces* sp. were finally isolated as CO₂-requiring extremely oligotrophic bacteria from soil samples collected from various places in Japan: isolates nos. 3 and 31 were isolated from mountainsides in Nara and Hyogo prefectures respectively, and isolate no. 31 was from a rice field in Aichi prefecture. Furthermore, a laboratory-stock *Streptomyces* strain showed growth similar to the isolates. Taken together with the fact that in the previous study, four laboratory-stock Rhodococcal strains showed oligotrophic growth similar to *R. erythropolis* N9T-4 isolated from crude oil, it is possible that such examples of this new type of oligotroph occur widely in nature and that certain properties of actinomycetes are responsible for oligotrophic growth. Recently, Watsuji et al. found that a syntrophic bacterium, *Symbiobacterium thermophilum*, was capable of marked mono-growth when gaseous CO₂ or bicarbonate ions were added to the medium, which might be due to a genetic defect in carbonic anhydrase.\(^\text{13}\) They also suggested that CO₂-dependence is significantly related to the issue of the unculturability of microorganisms. An atmospheric concentration of CO₂ was sufficient for the growth of the isolates in this study and for that of *R. erythropolis* N9T-4. Although further investigation is necessary to understand such oligotrophs, we think that these microorganisms have a novel and effective type of CO₂ fixation system or specific carbon metabolism that makes it possible for them to utilize CO₂ as their sole carbon source.

**References**


