

GROWTH INHIBITION OF OBLIGATELY OLIGOTROPHIC SOIL BACTERIA BY CARBOHYDRATES, AMINO ACIDS AND VITAMINS

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Obligately oligotrophic bacteria, 50 organisms, were isolated from soil. The sensitivity of them to D-glucose concentrations was classified in four types A, B, C and D. An oligotroph, K-27, classified in type A grew well at no addition of D-glucose to basal medium and was sensitive even to 0.01% D-glucose, and its growth was inhibited by various mono-carbohydrates at 0.1%. Oligotrophs classified in type C were presumed to be osmosensitive, and one of them, Y-28, was sensitive specifically to unnatural sugars, L-galactose and D-psicose. The addition of amino acids mixture or a single addition of amino acid and vitamin in a basal medium inhibited the growth of some oligotrophs. From these results it was discussed that there were different inhibition mechanisms by D-glucose concentrations, carbohydrates, amino acids and vitamins in these oligotrophs.

Key words; oligotrophs, soil bacteria, growth inhibition, glucose sensitivity, unnatural sugars.

Introduction

In a subcommittee meeting of the Japanese Society of Microbial Ecology, 1980, oligotrophs were defined as heterotrophic microorganisms which grew in a medium containing 1 mg organic carbon per liter⁽¹⁾. Oligotrophs have been isolated widely from soil, marine and other oligotrophic water environments. Moreover, oligotrophs unable to grow on nutrient rich media such as NB medium⁽²⁾ containing 1 % yeast extract, 1 % meat extract and 0.5% NaCl or LT medium⁽¹⁾ containing 0.5 % trypticase were conveniently defined as obligate oligotrophs⁽¹⁾.

Hattori and Hattori reported that the growth of soil DNB bacteria which were unable to grow on NB medium and might include oligotrophs was inhibited by inorganic salts, some amino acids, vitamins and organic acids⁽³⁾. Inhibitory effects of amino acids and organic acids on the growth of a few marine oligotrophs have been studied^(4, 5). But little has been known concerning organic substrates which inhibited the growth of obligately oligotrophic soil bacteria in nutrient rich media. Especially there was no report on inhibitory effects of carbohydrates. We presumed that important characters of obligate oligotrophs were their specific sensitivity to certain organic nutrients as well as their capability of utilizing very dilute nutrients.

Then in the present study we randomly isolated many obligately oligotrophic bacteria from soil and examined for their growth inhibition by various organic substrates such as D-glucose, other mono-carbohydrates including unnatural sugars, amino acids and vitamins at variable concentrations. And the results in this study will be expected to be a start of further investigations in the growth inhibition mechanisms of obligate oligotrophs.

In this report a term, "obligately oligotrophic bacteria" is often abbreviated to "obligate oligotrophs" or "oligotrophs."

Materials and Methods

Organisms. Fifty organisms isolated from soil were mainly used in this study. In some cases, *Escherichia coli* IFO 3544 and *Bacillus subtilis* IFO 13719 were used as standards of eutrophic bacteria.

Media and cultivation. Nutrient broth (NB) medium contained 10 g each of polypeptone and meat extract and 5 g of NaCl in one liter of deionized water and was adjusted to pH 7.0. Diluted nutrient broth (DNB) contained the same composition but at the strength of $1/10^2$ or $1/10^4$. Organic carbon content in NB/ 10^4 medium was calculated to be less than 1 ppm on the basis of analysis data⁽⁶⁾ of polypeptone and meat extract. Agar noble was used to solidify the medium for plate and stab cultures at 1.5% and 0.4%, respectively. Diluted nutrient broth (NB/ 10^2) medium was used as a basal medium for the growth experiments supplemented with mono-carbohydrates to ensure bacterial growth in control (not supplemented) cultures, whereas a synthetic medium was chosen for the evaluation of inhibitory effect of amino acids, vitamins and nucleic acid bases to control the content of these nutrients. Table I shows the composition of the synthetic medium, which is a modification of the basal medium used by Ishida *et al.*⁽⁷⁾. Deionized and distilled water was used for preparation of the media. Carbohydrates, amino acids, vitamins and nucleic acid bases were sterilized with filter and neutralized before filtration when necessary.

In the growth experiments the seed cultures were grown in NB/ 10^2 medium for 4~7 days at 27°C. Each 10 and 100 μ l of the seeds was inoculated in 5 and 50 ml of the main culture

Table I. Composition of Synthetic Medium

D-Glucose	100 mg	Na ₂ HPO ₄ ·12H ₂ O	200 mg
KH ₂ PO ₄	20 mg	MgSO ₄ ·7H ₂ O	5 mg
(NH ₄) ₂ SO ₄	100 mg		
Trace salt elements mixture ⁽¹⁾		0.1 ml	
Amino acids mixture ⁽²⁾		1.0 ml	
Vitamins mixture ⁽³⁾		1.0 ml	
Purine and pyrimidine bases mixture ⁽⁴⁾		1.0 ml	
Deionized and distilled water		1,000 ml	pH 7.0

(1) The mixture contains NaCl 500 mg, KCl 380 mg, CaCl₂·2H₂O 900 mg, MgSO₄·7H₂O 100 mg, K₂HPO₄ 100 mg, MnCl₂·4H₂O 40 mg, ZnCl₂ 40 mg, CuCl₂·2H₂O 1 mg, CoCl₂·6H₂O 0.5 mg, FeCl₃·6H₂O 2 mg and 1N-H₂SO₄ 1 ml per liter.

(2) The mixture contains each 1000 mg of 17 amino acids, 100 mg of cystine and 400 mg of tyrosine per liter.

(3) The mixture contains thiamine hydrochloride 96 μ g, nicotinamide 27 mg, riboflavin 9 mg, pyridoxine hydrochloride 2 mg, pantothenic acid 2.2 mg, biotin 6 μ g, *p*-aminobenzoic acid 1.7 mg, inositol 20 mg and cyanocobalamin 30.8 mg per liter.

(4) The mixture contains each 100 mg of adenine, guanine, xanthine, uracil, thymine and 4-amino-2-hydroxy-pyrimidine per liter.

media, respectively, and incubated statically for 10~15 days at 27°C.

Isolation and screening. Soil samples were obtained from farms, banks and gardens in Kagawa, Okayama and Tokushima prefectures. The soil samples, each 0.5 g, were shaken in 10 ml of sterilized water and filtered with Toyo filter paper No. 2. After suitable dilution, each 0.2 ml of the filtrates was spreaded onto plates of NB/10² and incubated at 27°C. Bacterial colonies developing after 5 days incubation up to 12 days were isolated into the NB/10² stab culture media and incubated at 27°C. The isolates grown in the stab cultures were inoculated in NB, NB/10² and NB/10⁴ media in double or triplicate. The organisms which could grow in NB/10² and NB/10⁴ but not in NB at least up to 7 days were selected as obligate oligotrophs. All isolates were confirmed to be bacteria by microscopic observations.

Assay methods. The degree of cell growth was measured by the increase in absorbancy at 600 nm of the culture fluid with light length of 5 cm. Viable cell numbers were measured by colony count method on NB/10² plate. Reducing sugar content in culture fluid was determined as D-glucose by the Nelson-Somogyi method⁽⁸⁾.

Chemicals. D-Tagatose and D-psicose were synthesized from galactitol by microbial transformation reactions^(9,10). Other carbohydrates were purchased from Sigma Chemical Company. Agar noble and yeast extract were purchased from Difco Laboratories. All other chemicals were obtained from Wako Pure Chemical Industry, Japan and were reagent grade.

Results

Isolation of obligate oligotrophs.

Four hundreds and seven colonies were isolated from 32 soil samples. Fifty isolates of them were selected as obligately oligotrophic bacteria because these organisms showed increase of viable cell number at 10~100 times during 10~20 days in NB/10⁴ but no turbidity in NB. Their taxonomic properties have not been tested yet.

Effect of D-glucose concentration on growth of obligate oligotrophs.

The fifty oligotrophs were grown in NB/10² medium containing 0~5% D-glucose for 10 days. The patterns of growth dependence of the organisms on D-glucose concentration were classified in the four types, A, B, C and D. The typical four patterns are shown in Fig. 1. Two organisms, K-27 and T-17, classified in type A showed the maximum growth without D-glucose and their growth decreased with increase of D-glucose concentration. The 13 organisms classified in type B showed the maximum growth at 1~3 % D-glucose. In 21 organisms classified in type C, their growth decreased markedly at 1~5 % D-glucose. The growth of 10 organisms classified in type D did not decrease with D-glucose concentration up to 5 %. The growth of the remaining four organisms was stimulated by the addition of D-glucose but the relationship between the growth degree and the D-glucose concentrations was not clear.

Growth inhibition of K-27 and V-50 by D-glucose.

Inhibitory effect of lower D-glucose concentrations on the growth of K-27 was tested and shown in Fig. 2. The growth was maximum at no addition of D-glucose and inhibited even by the addition of 0.01 % D-glucose. Since the reducing sugar content in the basal medium (NB/10²) was estimated to be about 10 mg/l as D-glucose, at least ten times higher concentration of D-glucose (or reducing sugar) affected its growth. The residual sugar amount in the every culture fluid after the incubation for 18 days little decreased except for in the 0.01 % D-glucose

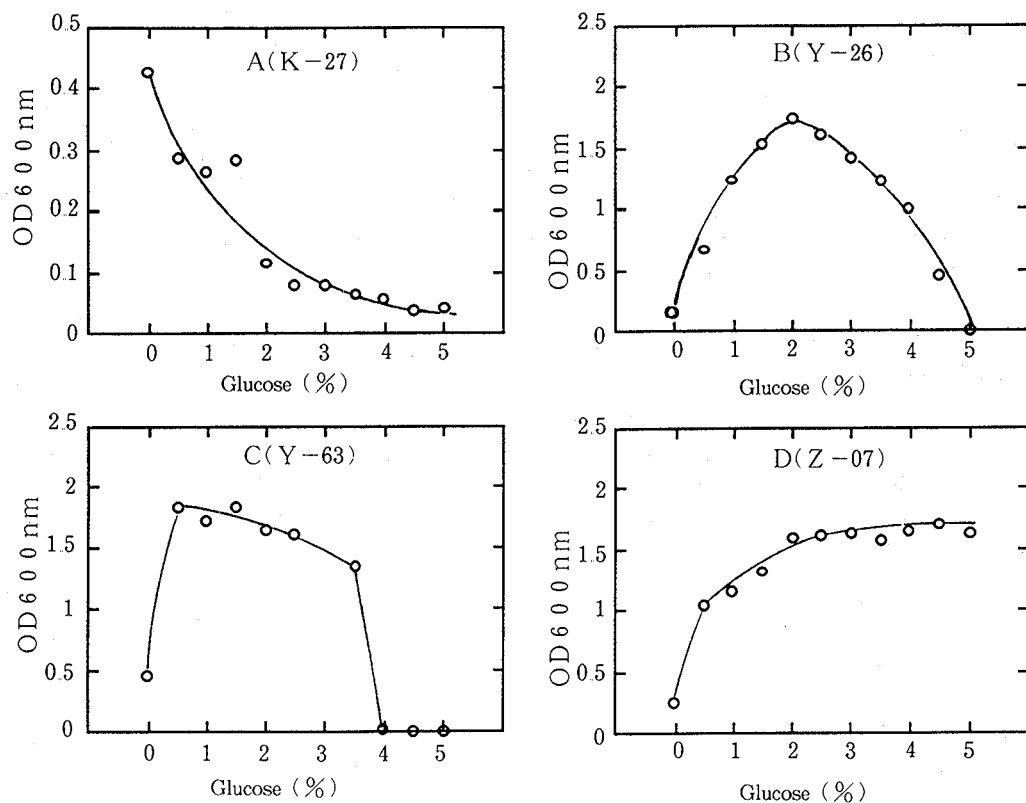


Fig. 1. Growth Dependence of Obligate Oligotrophs on D-Glucose Concentration. The organisms were grown in NB/10² containing D-glucose at various concentrations for 10 days.

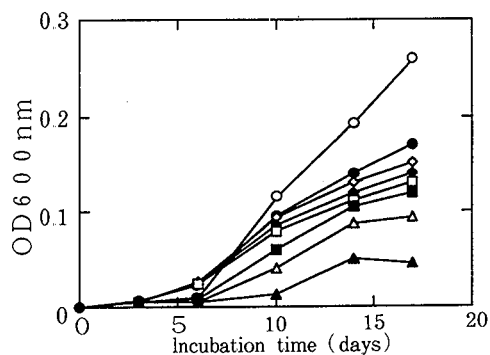


Fig. 2. Growth Inhibition of K-27 by D-Glucose. D-Glucose was added in NB/10² at concentrations of zero (○), 0.01 (●), 0.02 (◇), 0.05 (◆), 0.1 (□), 0.5 (■), 1.0 (△) and 2.0 (▲) %.

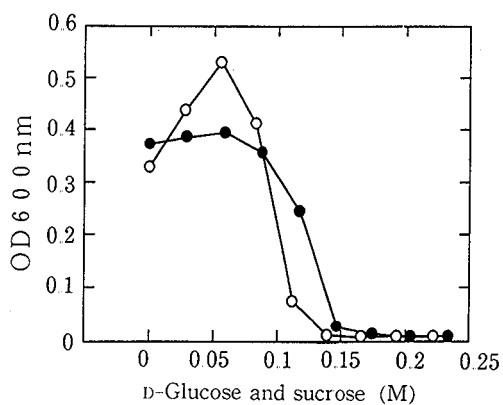


Fig. 3. Effect of D-Glucose and Sucrose Concentrations on the Growth of V-50. The organism was grown in NB/10² containing D-glucose (○) or sucrose (●) for 10 days.

culture where the concentration of reducing sugar declined to about 80 % of the initial.

As shown in Fig. 3, the organism, V-50, classified in type C showed the maximum growth at the addition of 0.056 M (1 %) D-glucose and the remarkable decrease of growth at the addition of more than 0.11 M (2 %). The growth was strongly inhibited also by the addition of a similar molar concentration of sucrose.

Effect of mono-carbohydrates on growth of obligate oligotrophs.

Eleven oligotrophs were chosen from each type of D-glucose sensitivity and grown in NB/10² supplemented with 0.1 % a single mono-carbohydrate for 10 days. The similar growth experiments were also carried out on *E. coli*. The degree of cell growth is shown in Table II. Many organisms grew well in media containing D-glucose, D-mannose, D-galactose, L-arabinose, D-xylose and D-fructose. Polyols a little stimulated the growth of some oligotrophs. All carbohydrates tested merely stimulated the growth of K-27 and in many cases various carbohydrates including some unnatural sugars inhibited its growth. The other oligotroph, T-17, classified in type A also showed a similar wide spectrum of carbohydrate sensitivity. And for the growth of V-50, only D-glucose of carbohydrates tested was a better substrate than the others at 0.1 %. L-Galactose, an unnatural sugar, stimulated the growth of some oligotrophs, but the other unnatural sugars were not good substrates for the growth of

Table II. Effect of Mono-Carbohydrates on the Growth of Obligate Oligotrophs

Carbohydrates (0.1%)	Relative growth** of oligotrophs and <i>E. coli</i>											
	K27	K25	V27	V50	Y26	Y28	Y31	Y63	Y66	Y96	Z07	<i>E. coli</i>
None	100	100	100	100	100	100	100	100	100	100	100	100
D-Glucose	28	150	120	190	300	300	400	340	120	230	510	200
D-Galactose	20	170	150	100	260	340	490	350	220	300	490	380
L-Galactose*	2	190	160	110	270	13	370	240	140	250	380	120
D-Mannose	2	110	140	110	240	420	390	220	130	260	670	220
D-Arabinose	110	340	110	110	110	130	110	90	120	120	120	210
L-Arabinose	130	110	170	100	280	350	440	360	160	370	620	220
D-Xylose	110	170	170	120	390	400	410	200	100	240	530	220
D-Ribose	96	80	150	110	100	120	100	100	180	120	130	250
D-Lyxose	7	170	140	100	140	140	160	95	150	120	150	130
D-Sorbitol	47	120	110	98	110	120	110	100	210	100	120	290
D-Mannitol	51	100	110	110	110	110	100	100	110	100	120	220
Galactitol	16	140	110	110	110	110	100	100	66	100	120	130
D-Arabitol	1	23	130	98	110	120	110	110	170	100	120	97
L-Arabitol	8	120	120	110	110	120	110	85	100	110	130	130
Xylitol	54	120	130	110	100	120	110	110	76	110	110	100
Ribitol	79	160	120	99	110	100	410	110	57	99	120	120
D-Fructose	25	130	120	100	190	230	340	150	230	150	550	190
D-Sorbose	1	100	110	100	100	120	100	100	73	110	120	120
L-Sorbose	27	130	110	100	96	110	100	100	80	100	100	70
D-Tagatose	92	150	110	100	100	120	100	100	92	110	130	110
L-Tagatose	76	150	110	100	110	110	100	10	79	110	120	110
D- Psicose	1	110	130	110	110	3	110	180	81	110	140	120

* The carbohydrates italicized are unnatural or rare sugars.

** The organisms were grown in NB/10² media supplemented with 0.1 % a single mono-carbohydrate for 10 days.

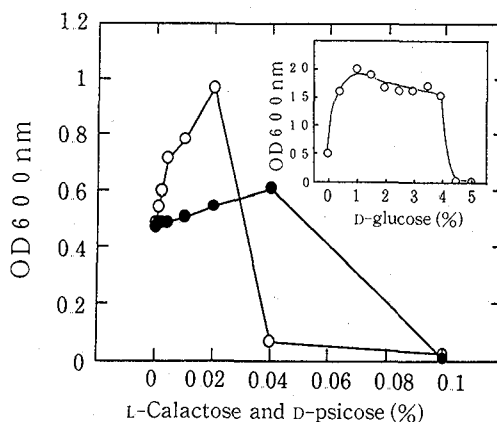


Fig. 4. Effect of L-Galactose, D-Psicose and D-Glucose Concentrations on the Growth of Y-28. The organism was grown in NB/10² containing L-galactose(○), D-psicose(●) and D-glucose for 10 days.

many oligotrophs.

Bacterial strain, Y-28, classified in type C is an interesting organism at the viewpoint of response to mono-carbohydrates. This isolate grew well in the presence of D-glucose, D-galactose, D-mannose, L-arabinose, D-xylose and D-fructose while it was markedly sensitive to specific unnatural sugars, L-galactose and D-psicose. A similar was true for the strain Y-63 whose growth was specifically inhibited by L-tagatose. Response of these organisms to unnatural sugars somewhat differs from that of K-27 whose spectrum of sensitivity to mono-carbohydrates was not specific. Effect of concentrations of these unnatural sugars and D-glucose on the growth of Y-28 is shown in Fig. 4. The growth was strongly inhibited by the addition of 0.04 % L-galactose or 0.1 % D-psicose. Based on a molar concentration, these unnatural sugars were 45 to 113 times more inhibitory to the growth of Y-28 than D-glucose.

Effect of amino acids mixture on growth of obligate oligotrophs.

Fifty obligate oligotrophs were grown for 10 days in the synthetic medium supplemented with an amino acids mixture which contained each 100 mg/l (100 times stronger than the control) of 17 amino acids, 50 mg/l (125 times stronger) of tyrosine and 10 mg/l (100 times stronger) of cystine. But cysteine was not contained in the mixture because it inhibited the growth of various bacteria strains including oligotrophs and eutrophs as described subsequently. *E. coli* and *B. subtilis* were also grown in the same medium for comparison. By the supplement of amino acids mixture, the growth was stimulated remarkably (more than 200 % as compared with the control without the supplement) for 16 oligotrophs and *E. coli*, moderately (120~200 %) for 19 oligotrophs and *B. subtilis*. On the other hand, the growth was inhibited strongly (less than 50 %) by the supplement for 11 oligotrophs and moderately (50~80 %) for 2 oligotrophs. In the residual two the relative growth was 80~120 % of the control.

Growth inhibition of obligate oligotrophs by a single amino acid.

Three obligate oligotrophs whose growth was strongly inhibited by the supplement of

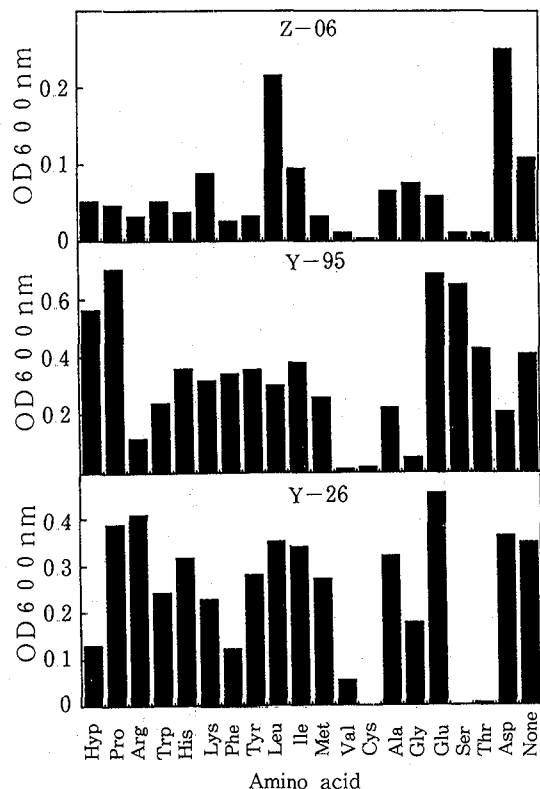


Fig. 5 Effect of a Single Amino Acid on the Growth of Y-26, Y-95 and Z-06. The organisms were grown in the synthetic media containing alone 5 mM of each amino acids except for 0.2 mM of tyrosine for 10 days.

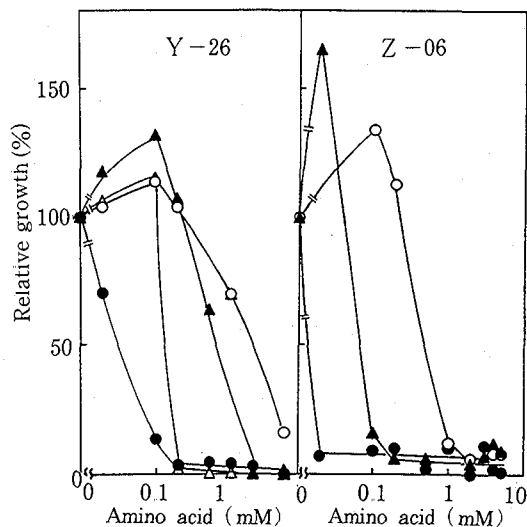


Fig. 6. Inhibitory Effect of Amino Acid Concentrations on the Growth of Y-26 and Z-06. The organisms were grown in the synthetic media containing a single amino acid at various concentrations for 10 days. Cysteine, ●; valine, ○; threonine, △; serine, ▲.

amino acids mixture were examined for their sensitivity to a single amino acid. The synthetic medium was used to be supplemented with 5 mM of a single amino acid except for 0.2 mM of tyrosine. Figure 5 shows the effect of a single amino acid on the growth of Y-26, Y-95 and Z-06. The oligotrophs, Y-26 and Y-95 were specially sensitive to only 4 or 5 amino acids while Z-06 was sensitive to more amino acids. Cysteine and valine inhibited strongly the growth of three organisms. Serine and threonine were inhibitory for Y-26 and Z-06, glycine and arginine for Y-95 and Z-06. The growth of two eutrophs, *E. coli* and *B. subtilis* was inhibited only by cysteine.

Figure 6 shows the inhibitory effect of concentrations of some amino acids on the growth of Y-26 and Z-06. Both oligotrophs were most sensitive to cysteine and their growth was inhibited by 0.02 mM of cysteine. Serine of 0.2 mM and threonine of 0.1 mM inhibited strongly the growth of Y-26 and Z-06, respectively. The growth inhibition by valine was observed at concentrations of more than 1.0 mM.

Effects of vitamins, purine and pyrimidine bases on growth of obligate oligotrophs.

No growth inhibition of oligotrophs, *E. coli* and *B. subtilis* was observed by the supplement of nine vitamins mixture or nucleic acid bases mixture at 100 times of the concentration in Table I. Three organisms, K-27, K-013 and K-034 were examined for the growth inhibition

Table III. Effect of Vitamins on the Growth of K-27, K-013 and K-034

Vitamins	Concentration (mg/l)	Relative growth* of oligotrophs		
		K-27	K-013	K-034
None	0	100	100	100
Thiamine hydrochloride	0.0096	1400	140	110
Nicotineamide	0.27	10	120	53
Riboflavin	0.9	180	140	26
Pyridoxine hydrochloride	0.2	220	2	59
Pantoteinic acid	0.22	70	130	28
Biotin	0.0006	290	140	70
<i>p</i> -Aminobenzoic acid	0.17	60	110	41
Inositol	2.0	20	130	27
Cyanocobalamin	3.0	30	140	93
All vitamins	6.7702	4800	120	130

* The oligotrophs were grown in the synthetic media supplemented with a single vitamin for 10 days

by a single supplement of nine vitamins. The growth of K-27 was most stimulated by the supplement of vitamins mixture and the growth of the latter was least stimulated. Table III shows the effect of a single vitamin on the growth of three organisms. Nicotineamide, riboflavin, pyridoxine hydrochloride, pantoteinic acid, *p*-aminobenzoic acid, inositol and cyanocobalamin showed the inhibitory effect on the growth.

Discussion

It has been generally thought that D-glucose is a good carbon source for the growth of most microorganisms. Ohta and Hattori⁽¹¹⁾ discussed that D-glucose was one of the most important carbon source for DNB bacteria. But the growth of a considerable number of obligately oligotrophic bacteria isolated in this study was inhibited by D-glucose of a certain concentration as shown in Fig. 1. Among them, organisms classified in type C may be osmosensitive to a certain concentration of sugars rather than sensitive to D-glucose itself, since the strong growth inhibition in V-50 belonging to type C appeared at a similar molar concentration of D-glucose and sucrose (Fig. 3).

The inhibition mechanisms in K-27 classified in type A are presumed to be different from those in oligotrophs in type C. It is interesting what mechanisms of the strong growth inhibition by D-glucose are in K-27 cells, whose growth was inhibited even by 0.01% D-glucose (Fig. 2). The growth inhibition by D-glucose has been rarely reported in bacteria except for in anaerobes such as *Clostridium*^(12,13) and *Bacteroides*⁽¹⁴⁾. The strain K-27 was more sensitive (100 times or more) to the concentration of D-glucose than the anaerobes and showed very wide spectrum of growth inhibition by mono-carbohydrates (Table II). We now attempt to elucidate the inhibition mechanisms by D-glucose in K-27.

The effect of unnatural sugars on growth of microorganisms has been little tested because these sugars are available with difficulty, especially D-tagatose and D-psicose are so. As we expected, many unnatural sugars were little effective to enhance the growth of many oligotrophs tested (Table II). Besides K-27 which was sensitive to various unnatural sugars,

0.04% L-galactose and 0.1% D-psicose specifically inhibited the growth of Y-28 (Fig. 4) which belonged to type C concerning to sensitivity to D-glucose. Therefore, it is suggested that the inhibition mechanism by unnatural sugars is different from that by D-glucose in Y-28 cells. In contrast, L-galactose, unlike other unnatural sugars, stimulated the growth of some oligotrophs (Table II). No report has been found on the metabolism of L-galactose by microorganisms. We expect that these results in oligotrophs will be compared with those in many eutrophs other than *E. coli*.

One of the reasons for which obligate oligotrophs cannot grow in NB medium is expected to be in the growth inhibition by high concentrations of certain amino acids contained in the medium. Hattori and Hattori⁽³⁾ showed that some amino acids of 10 mM inhibited the growth of soil DNB bacteria. Akagi *et al.*⁽⁴⁾, Martin and Macleod⁽⁵⁾ reported that the growth of marine oligotrophs was inhibited by alanine or phenylalanine of 1000 mg carbon per liter. We showed that the growth of the obligate oligotrophs isolated in this study was inhibited by a single addition of some amino acids at lower concentrations than those described above (Fig. 6). Thus growth inhibition of bacteria by amino acids at similar concentrations to our study was observed in *Escherichia coli*^(15,16), *Thiobacillus neapolitanus*⁽¹⁷⁾ and *Agmenellum quadruplicatum*⁽¹⁸⁾. When cysteine was oxidized by oxygen⁽¹⁹⁾ or heated at pH 6⁽²⁰⁾, it was reported that the antibacterial compounds were formed from cysteine. In this study it was not examined whether such compounds were formed.

It is a well known phenomenon that growth of some bacteria is inhibited by unbalanced concentrations of certain natural L-amino acids⁽²¹⁾. Thus growth inhibition is often recovered by other amino acids, for example, in *E. coli*⁽²²⁾ and *A. quadruplicatum*⁽¹⁸⁾. And in DNB bacteria it was reported that their serine-sensitivity was recovered by coexisting with threonine or a mixture of leucine and valine⁽³⁾. In our obligate oligotrophs thus recovery by other amino acids has not been observed yet. The growth of three oligotrophs tested was strongly inhibited not only by a single amino acid at very low concentrations but by amino acids mixture (Fig. 6). Therefore, it is suggested that growth inhibition of some oligotrophs results from balanced amino acids through unknown mechanisms as well as from unbalanced composition of amino acids. In contrast, excess vitamins when these were supplemented solely showed an inhibition similar to the effect by unbalanced composition (Table III).

Unfortunately, the relationship between sensitivity to the organic compounds tested and a physiological importance of such sensitivity was not clear. But we suggested that there were different types in the growth inhibition of obligately oligotrophic soil bacteria by D-glucose concentrations, other mono-carbohydrates, amino acids and vitamins. In further studies the characteristic inhibition mechanisms will be investigated in certain oligotrophs.

Oligotrophs were reported not only to be predominant in oligotrophic conditions such as in open ocean⁽²³⁾, in periphyton at an oligotrophic river⁽²⁴⁾ and in NB/10⁴ medium⁽²⁵⁾ but to coexist widely in general soil and even in fertile soil⁽¹¹⁾ together with eutrophs. Thus specific growth inhibition by certain organic nutrients is expected to be a strategy for survival which obligate oligotrophs obtained during the process of evolution.

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偏性低栄養土壤細菌の糖質、アミノ酸およびビタミン による生育阻害

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土壤から偏性低栄養細菌を50株分離した。これらはグルコース各種濃度に対する感受性から4つのタイプに類別された。K-27株は0.01%のグルコースによってさえ生育阻害を受け、その他0.1%の各種単糖にも感受性を示した。Y-28株は非天然糖であるL-ガラクトースおよびD-ブシコースに対して特異的な感受性を示した。アミノ酸混液の添加あるいはアミノ酸、ビタミンの各単独添加によっても生育阻害を受けるものもあった。これらの結果から、偏性低栄養細菌には特定の有機栄養物に対して各種の特異的な生育阻害機構が存在することが示唆された。