Competition For Inorganic Substrates Among Chemoorganotrophic And Chemolithotrophic Bacteria

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Abstract. In aerobic enrichment experiments with a chemostat, using phosphate-limited lactate medium, a *Spirillum* sp. predominated at the lower range of dilution rates. At the higher dilution rates an (chemoorganotrophic) unidentified rod-shaped bacterium came to the fore. The same result was obtained in competition experiments with pure cultures of the two bacteria. Growth parameters were: Rod, $\mu_{max}=0.48$ hr^{-1}, $k_s(PO_4^{3-})=6.6\times10^{-8}$ *M*; *Spirillum*, $\mu_{max}=0.24$ hr^{-1}, $k_s(PO_4^{3-}) = 2.7 \times 10^{-8}$ *M*. The *Spirillum* grew faster than the rod at low dilution rates, not only under phosphate-limitation but also in K^+-,Mg^{2+}-, NH_4^+-, aspartate-, succinate-, and lactate-limited cultures. Both organisms showed little substrate specificity and could utilize a similar range of carbon and energy sources. The results support the view that part of the diversity among bacteria in the natural environment is based on selection toward substrate concentration. Another set of competition experiments was carried out with pure cultures of two marine obligately chemolithotrophic colorless sulfur bacteria, *Thiobacillus thioparus* and *Thiomicrospira pelophila*. *Tms. pelophila* outgrew *T. thioparus* at low dilution rates under iron limitation, while the reverse was true at high dilution rates. It is concluded that the relatively fast growth of *Tms. pelophila* at low iron concentration may explain its higher sulfide tolerance. Organisms showing a selection advantage at very low concentrations of limiting substrates appear to have a relatively high surface to volume ratio.

Introduction

In natural environments the rate of bacterial growth is frequently limited by the concentration of an essential nutrient. The relation between specific growth rate (μ) and the concentration of a limiting substrate (s) is empirically described by [15]:

$$\mu = \mu_{max} \frac{s}{k_s + s} \qquad (1)$$

where μ_{max} is the maximum specific growth rate and k_s is a saturation constant, numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\mu_{max}$.

The result of competition of a mixed bacterial population for a single growth-limiting substrate depends on the μ – s relationships of the bacteria involved [16]. Results obtained with mixed populations of aerobic chemoorganotrophic bacteria limited by a C– and energy source in the chemostat were described by Jannasch [6] and Harder and Veldkamp [5]. It was shown that in

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natural waters selection toward the concentration of growth-limiting C– and energy sources is quite common. At extremely low concentrations different chemoorganotrophic organisms came to the fore than found at higher concentrations. Organisms of the former type were characterized by a relatively low k_s and μ_{max}.

Competition for inorganic substrates was studied in the chemostat by Meers and Tempest [4] and Meers [13] with chemoorganotrophic organisms derived from culture collections. Only on one occasion crossing μ–s curves were obtained. The example was obtained with potassium- and magnesium-limited mixed cultures of *Bacillus subtilis* and *Torula utilis* [13].

The purpose of the present investigation was to extend our knowledge on selection toward concentration of inorganic substrates in bacteria occurring in the same habitat. Results will be described of competition for phosphate among aerobic chemoorganotrophic freshwater bacteria and of competition for iron between chemolithotrophic sulfur bacteria occurring in marine mud flats.

Materials and Methods

Organisms. The chemolithotrophic *Thiomicrospira pelophila* and a marine strain of *Thiobacillus thioparus* were maintained and cultivated at 25°C as described by Kuenen and Veldkamp [10]. A chemoorganotrophic *Spirillum* sp. and a chemoorganotrophic unidentified rod-shaped organism were isolated from aerobic mixed chemostat cultures which had been inoculated with pond water (see below). They were purified, maintained, and cultured at 25°C in basal medium (see below), supplemented with 0.1% (w/v) sodium lactate, with or without 1% (w/v) agar. Agar-slant cultures were transferred every 3rd week.

Media. For cultivation of *T. pelophila* and *T. thioparus* an (autotrophic) medium of the following composition was used (%, w/v): NaCl, 2.5; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7\ H_2O$, 0.15; $CaCl_2 \cdot 2\ H_2O$, 0.03; $Na_2S_2O_3 \cdot 5\ H_2O$, 0.4; KH_2PO_4, 0.036; K_2HPO_4, 0.012; vitamin B_{12}, 1.5×10^{-6}; trace element solution (containing EDTA) [18], 1 ml/liter of medium. For media which contained growth-limiting concentrations of iron, the same trace element mixture without iron was used. The chemicals were dissolved in deionized, distilled water. The final pH was 6.8. Media were sterilized by autoclaving for 20–40 min at 120°C. Phosphates were sterilized separately. Media to be used for competition experiments were sterilized by filtration through membrane filters (Sartorius, Göttingen, W. Germany, 0.22μm pore size) as described previously [11]. As most chemicals contain traces of iron salts, the growth-limiting concentration of iron was determined empirically by adjusting its concentration (with either $FeCl_3$ or $FeSO_4$ solution) in the medium reservoir until dry weight of the culture was between 30–50% of that obtained with excess iron in the medium. It was necessary to determine this growth-limiting concentration of iron in the medium for each dilution rate tested. At higher dilution rates a higher concentration of iron in the medium was needed to maintain the same cell density. The reason for this phenomenon is not known. It must be stressed, however, that at each dilution rate tested, iron was the growth-limiting factor since increasing or decreasing its concentration in the medium reservoir accordingly increased or decreased the cell density of the culture.

For cultivation of the *Spirillum* sp. and the rod-shaped bacterium a basal medium of the following composition was used (%, w/v): NH_4Cl, 0.02; K_2HPO_4, 0.03; $MgSO_4 \cdot 7\ H_2O$, 0.003; $FeCl_3 \cdot 6\ H_2O$, 5×10^{-4}; $CaCl_2 \cdot 2\ H_2O$, 7×10^{-4}; trace elements solution, 1 ml per liter of medium. Routinely 0.1% (w/v) sodium lactate was used as the only carbon and energy source. In two experiments lactate

was replaced by sodium aspartate (0.1%, w/v) or sodium succinate (0.2%, w/v). Final pH of the medium was 7.0. Media were sterilized by autoclaving for 30–40 min at 120°C. Phosphate was sterilized separately.

The composition of the media used for competition experiments was essentially the same. Growth-limiting concentrations of the substrate under investigation were chosen such that at least a fourfold increase in cell density could be obtained by increasing the concentration of the growth-limiting substrate. In the different media the following concentrations were used for the respective growth-limiting substrates (%, w/v): sodium lactate, 0.025; sodium aspartate, 0.02; sodium succinate, 0.045; NH_4Cl, 2×10^{-3}; $^{K}{}_{2}HPO_4$; 7×10^{-4}, $MgSO_4{\cdot}7\,H_2O$, 2×10^{-4}; KCl, 8×10^{-5}. If needed, 1% (w/v) agar was used to solidify the media.

Chemostat. In all experiments all-glass equipment was used as described by Veldkamp and Kuenen [17]. Medium was added by a tubingpump (LKB Varioperspex 12000, Bromma, Sweden). The pH was maintained at the desired value with a scanning pH-stat described by Kuenen *et al*. [9]. For neutralization of cultures of *T. pelophila* and *T. thioparus*, a 0.5 M Na_2CO_3 solution was used. Cultures of the two heterotrophic bacteria, and of related enrichment cultures (selection experiments, see below), were neutralized with O.1 N sulfuric acid, except under phosphate limitation, in which case lactic acid (0.5 N) was used.

Counting Procedures. Direct counting of bacterial cells was performed in a Bürker-Türk counting chamber of 0.01 mm depth. It proved possible to count the spirillum-shaped *T. pelophila* and the rod-shaped *T. thioparus* separately in mixed cultures, which allowed estimation of the ratio of the two organisms. Similarly, numbers and ratios of the *Spirillum* sp. and the heterotrophic rod-shaped bacterium could be determined in mixed cultures.

Good agreement was obtained between direct counts and numbers of colonies on agar plates. Viable counts were usually not lower than 70% of the total counts. For the heterotrophs, 0.1% lactate plates were used, while for the chemolithotrophs, 0.5% thiosulfate plates were employed.

Enrichment and Isolation of Chemoorganotrophic Bacteria. Chemostats, running at the desired dilution rate, were inoculated with a sample from a little pond near the laboratory. The sample was filtered through a 2μm membrane filter (Sartorius) in order to remove debris and larger organisms such as protozoa. Phosphate was the growth-limiting factor in the medium. After at least 5 volume changes, dominant organisms were isolated from cultures run at different dilution rates by plating out dilution series of samples on 0.1% lactate agar.

Competition Experiments with Pure Cultures. Pure cultures of the chemolithotrophic or heterotrophic bacteria were grown in separate chemostats at a given dilution rate and under growth limitation by the substrate to be investigated. After a steady state had been established, cultures were mixed at approximately equal cell densities. All competition experiments with *T. pelophila* and *T. thioparus* were performed according to this procedure. For the heterotrophs this procedure was adopted only for phosphate limitation. In all other competition experiments fresh, exponentially growing batch cultures, pregrown in a rotary-shaker on the appropriate substrate, were mixed at approximately equal cell densities in the chemostats running at the desired dilution rate.

Maximum Specific Growth Rate. The μ_{max} values of the heterotrophic *Spirillum* sp. and the rod-shaped bacterium were determined essentially as described by Harder and Veldkamp [4]. The μ_{max} values for *T. pelophila* and *T. thioparus* were estimated from the respective critical dilution rates in the chemostat [10].

Rapid Sampling. For the determination of steady-state concentrations of the growth-limiting substrate rapid sampling and removal of bacteria is obligatory. This was done as follows. A sterile membrane filter (Millipore, Bedford, Massachusetts, U.S.A., 0.45 μm pore size, prewashed with

Table 1
Surface-to-Volume Ratios of Four Couples of Chemostat-Grown Spiral- and Rod-Shaped Bacteria Competing for the Growth-limiting Substrate.[a]

Organism	Limiting substrate	Specific growth rate (hr^{-1})	Average length (μm)	Average diameter (μm)	Surface/Volume ($\mu^{-1}m$)
1. *Pseudomonas* sp.[b]	Lactate	0.1	2.9	1.1	4.3
Spirillum sp.	Lactate	0.1	3.5	0.55	8.0
2. *Pseudomonas* sp.[c]	Lactate	0.15	2.3	1.0	4.9
Spirillum sp.	Lactate	0.15	2.6	0.66	6.8
3. Unidentified rod[d]	Phosphate	0.2	2.8	1.1	4.3
Spirillum sp.	Phosphate	0.2	3.8	0.6	7.2
4. *Thiobacillus thioparus*[d]	Thiosulfate	0.1	2.0	0.4	11
Thiomicrospira pelophila	Thiosulfate	0.1	2.5	0.2	21

[a] Surface to volume ratios were calculated from the dimensions of the bacterial cells, treating them as small cylinders. Dimensions of bacterial cells were estimated from photomicrographs and from electron micrographs. For further details, see Materials and Methods.

[b] Harder and Veldkamp, unpublished observations

[c] Matin and Veldkamp, in preparation

[d] This report

double distilled water) mounted in a plastic filter holder (Millipore) was connected to a thin (1mm i.d.) tube leading into the culture liquid. To the open end of the filter holder a syringe was attached. In less than 60 sec approximately 50 ml of culture filtrate could be obtained by operating the syringe. To minimize substrate consumption and to prevent clogging of the filter during sampling, the cell density was lowered by reducing the concentration of the growth-limiting substrate in the reservoir by a factor of 10. It could be calculated that under these conditions consumption of growth-limiting substrate during sampling was less than 10% of the total substrate present in the filtrate.

Surface-to-Volume Ratios. The surface-to-volume ratios (s/v) of the bacterial cells were calculated from their dimensions as estimated both under the phase-contrast microscope (measured with a calibrated micrometer or from photomicrographs) and from electron micrographs. Dimensions as given in Table 1 are averages of at least 100 measurements. Deviations of the average diameter were never more than 10%. The length varied by 20%. In the calculation for the (s/v) the bacterial cells were treated as small cylinders. The (s/v) is then given by (s/v) = (2/R) + (2/L), where R is the radius (half of the diameter) and L is the length of the bacterium. In all bacteria the R is small relative to L and consequently the diameter of the bacterium is crucial for its (s/v).

Miscellaneous. Phosphate was determined according to a slightly modified method of Murphy and Riley (quoted in [2]). Total iron (Fe^{2+} and Fe^{3+}) was measured with the bathophenanthroline method (Lee and Stumm, quoted in [2].

Results

Competition for Phosphate in Aerobic Chemoorganotrophic Bacteria

An enrichment experiment was carried out with two phosphate-limited chemostats, connected to the same medium reservoir and run at dilution rates (D) of 0.03 and 0.3 hr^{-1}, respectively. The chemostats were inoculated with pond water, and after 5 volume changes a *Spirillum* sp. had come to the fore at a low dilution rate, whereas a Gram-negative rod-shaped organism dominated at a high dilution rate. Pure cultures of both organisms could easily be obtained and competition experiments were carried out at 5 dilution rates using inocula with approximately equal numbers of both organisms. A typical result is shown in Fig. 1. The k_s value of the rod was determined by growing the organism under steady-state conditions in a phosphate-limited chemostat at dilution rates near μ_{max} and measuring the phosphate concentration in the culture (see Materials and Methods). The concentration was $4.1 \pm 0.9 \times 10^{-7} M$, at $D = 0.42$ hr^{-1}. Since μ_{max} was known from batch culture experiments (0.48 hr^{-1}, see Materials and Methods), k_s could be calculated from Eq. (1) as $6.6 \pm 1.3 \times 10^{-8} M$ phosphate. The steady-state phosphate concentrations in continuous cultures of the *Spirillum* sp. were too low to be detectable. However, the k_s of the *Spirillum* sp. could be calculated from its known μ_{max} and from the crossing point of the μ–s curves of both organisms. From the outcome of the competition experiments this point could be estimated as being close to $D = 0.07$ hr^{-1}. At the crossing point the growth rate ($\mu = D$) and substrate concentration in the chemostat are equal for both organisms and thus the phosphate concentration at the crossing point could be calculated from Eq. (1) using the known parameters of the rod. This concentration was $1.1 \times 10^{-8} M$. Substituting this concentration in Eq. (1), and using

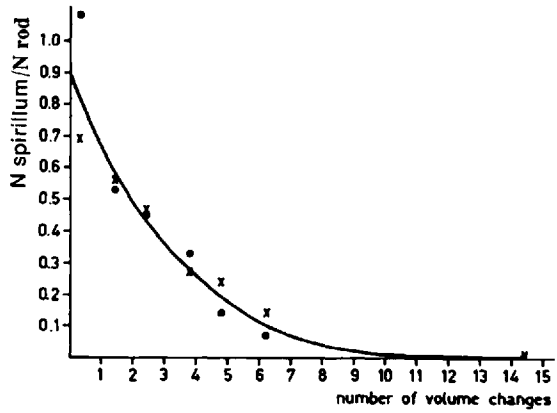

Fig. 1. Change in ratio of cell numbers of *Spirillum* sp. and rod-shaped bacterium as a function of the number of volume changes in the chemostat. The bacteria were grown in a phosphate-limited medium, using lactate as C− and energy source. For further details, see Materials and Methods. D = 0.3 hr^{-1}; T = 25°C; x: viable count; •: direct count.

the known μ_{max} of the *Spirillum* (0.24 hr^{-1}, see Materials and Methods), the k_s of the *Spirillum* was calculated as 2.7 × 10^{-8} M. The μ–s relations of both organisms are shown in Fig. 2 (upper right).

As the phosphate concentration in the pond water from which the organisms were derived was just above the detection level (5 × 10^{-8} M) it is feasible that phosphate was growth-limiting in the natural environment.

The surface-to-volume ratios of the two organisms in phosphate-limited chemostats were measured at D = 0.2 hr^{-1} (see Materials and Methods). They were 7.2μm^{-1} for the *Spirillum* sp. and 4.3μm^{-1} for the rod-shaped organism (Table 1).

Table 2
Maximum Specific Growth Rates (μ_{max}) in Basal Medium with Different C- and Energy Sources.[a]

Substrate	*Spirillum* sp. μ_{max}(hr^{-1})	Rod μ_{max}(hr^{-1})
Na-lactate	0.24	0.48
Aspartate	0.17	0.38
Glutamate	0.26	0.33
Na-succinate	0.25	0.35

[a] The μ_{max} values were determined from logarithmic plots of exponential growth in batch culture by standard methods (see Materials and Methods). pH = 7.0; Temp. = 25°C.

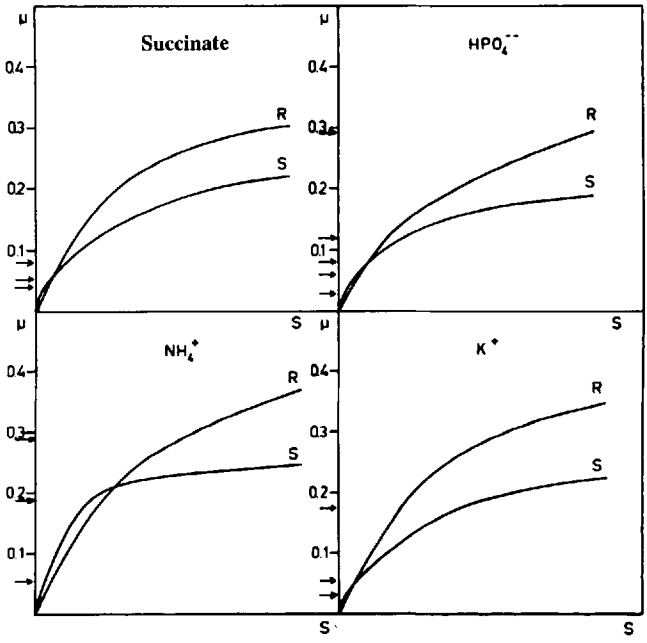

Fig. 2. Specific growth rate (μ) of *Spirillum* sp. (S) and rod-shaped bacterium (R) as a function of the growth-limiting substrate as indicated. The results were obtained from competition experiments carried out in the chemostat. Lactate was the C− and energy source in experiments with inorganic ion-limitations (PO_4^{3-}, $HHb54^+$, and K^+). Temperature 25°C. Arrows indicate dilution rates at which competition experiments were carried out. For maximum specific growth rates (μ_{max}) in succinate medium (upper left) and lactate medium (other graphs) see Table 2. In phosphate limited-(lactate) medium the substrate saturation constant (k_s) was $k_s(PO_4^{3-}) = 2.7 \times 10^{-8} M$ for the *Spirillum* and $k_s(PO_4^{3-}) = 6.6 \times 10^{-8} M$ for the rod. For further details, see text and Materials and Methods.

Subsequently both organisms were exposed to competition for other growth-limiting subtrates. The results for succinate, potassium, and ammonia-N are shown in Fig. 2. The k_s values for these substrates were not determined. The curves are schematic and based on μ_{max} determinations and results of competition experiments at various dilution rates. Similar results were obtained for Mg^{2+}, -aspartate-, and lactate-limitation.

Substrate specificities of both organisms, as determined by testing growth in a liquid mineral medium supplied with 20 C− and energy sources, showed only minor differences. Glucose could be utilized by the *Spirillum* sp., but not by the rod, although both organisms grew on gluconate. The rod could grow on glycine which was not utilized by the *Spirillum* sp. In Table 2 differences in μ_{max} values are shown for four substrates when used as single C− and energy sources.

Competition for Iron in Chemolithotropic sulfur Bacteria

Another example for competition for inorganic substrates was found during a study on the ecological niches of two closely related colorless sulfur bacteria. The two obligately chemolithotropic bacteria, *Thiomicrospira pelophila,* and *Thiobacillus thioparus,* were isolated from the same samples taken from intertidal mudflats of the Dutch Waddenzee [10]. No differences could be detected in the energy and carbon metabolism of the two organisms [7, 11]. Since it was highly unlikely that both organisms would have the same functional status in their habitat, a study was made of a number of factors which might affect the growth of both organisms. It was found that the sulfide tolerance of *T. pelophila* was higher than that of *T. thioparus* [10]. The general phenomenon of toxicity of sulfide is well known. High concentrations of sulfide even inhibit growth of photosynthetic bacteria, which use sulfide as an electron donor [1, 3]. As one of the possibilities it was postulated that the inhibitory effect of sulfide could be caused by low concentrations of iron due to the low solubility product of ferrous sulfide [3,7]. This prompted an experiment to test the effect of the concentration of available iron on the growth rates of *T. pelophila* and *T. thioparus*. Therefore, the two bacteria were grown in a mixed culture in a chemostat with complexed iron as the growth-limiting factor. The result of the experiments is represented in Fig. 3, which shows crossing substrate saturation curves for the two organisms. Since the iron concentration in the chemostat was extremely low, it was not possible to determine its absolute value. The curves were drawn on the basis of the known μ_{max} values and the outcome of the competition experiments. As can be seen, *T. pelophila* even became dominant at growth rates near to its maximum specific growth rate. At all dilution rates tested, the iron in the medium was the growth-limiting factor, since adding iron to the chemostat resulted in an

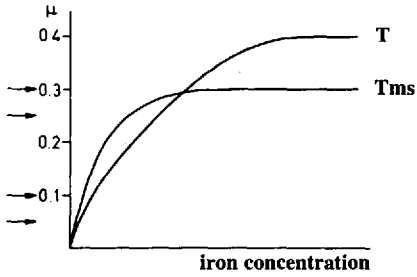

Fig. 3. Specific growth rate (μ) of *Thiomicrospira pelophila* (Tms) and *Thiobacillus thioparus* (T) as a function of the growth-limiting iron concentration. The results were obtained from competition experiments carried out in the chemostat. Bacteria were grown in a thiosulfate-minerals medium at 25°C. Arrows indicate dilution rates at which competition experiments were carried out. μ_{max}(Tms) = 0.35 hr^{-1}; μ_{max} (T) = 0.45 hr^{-1}. For further details, see text and Materials and Methods.

immediate increase of cell mass, both in pure and mixed cultures. Spent medium of iron-limited chemostat cultures of *T. pelophila* did not inhibit growth of pure cultures of *T. thioparus* in batch culture. Therefore the outcome of the competition experiments in favor of *T. pelophila* is very likely not due to production of a substance by this organism which inhibits the growth of *T. thioparus*. It should be realized that these experiments were carried out in the presence of excess oxygen and thiosulfate so that it remains unknown whether the iron in the medium was taken up as ferric or as ferrous irons.

The surface-to-volume ratio of both organisms, grown in a thiosulfate-limited chemostat at a dilution rate of 0.1 hr^{-1}, was estimated from both [light and electron microscopy] as being approximately $21\mu m^{-1}$ for *T. pelophila* and $11\mu m^{-1}$ for *T. thioparus* (Table 1). (For further details see Materials and Methods.)

Discussion

The experiment in which a mixed population obtained from pond water was exposed to different concentrations of growth-limiting phosphate in the chemostat showed that dependent on the phosphate concentration, different organisms came to the fore. The result thus was similar to previous experiments in which competition for C— and energy sources was studied in chemoorganotrophic bacteria [5,6], in that it was found that in natural waters organisms occur which have a selective advantage at extremely low concentrations of growth-limiting substrates. The maximum specific growth rates of these organisms are comparatively low; therefore they never appear in the classic batch culture enrichments.

The *Spirillum* sp. which came to the fore at extremely low phosphate concentrations not only grew faster than the rod-shaped organism under these conditions, but also outgrew the latter bacterium in a range of very low concentrations of K^+, NH_4^+, and succinate (Fig. 2). Similar results were obtained with Mg^{2+}, lactate, and aspartate. As yet it is not known, however, whether other organisms are present in its natural environment which may grow even faster at low concentrations of these substrates, other than phosphate.

As in previous competition experiments with chemoorganotrophs [5] the organisms isolated from phosphate-limited chemostats showed little substrate specificity as far as C— and energy sources are concerned. The indications thus are that part of the diversity among chemoorganotrophic bacteria in natural waters can be explained in terms of selection toward concentration of both organic and inorganic nutrients.

Similar considerations hold for the chemolithotrophic colorless sulfur bacteria studied. Both *Thiobacillus thioparus* and *Thiomicrospira pelophila* were

isolated from the marine mud flats, in which both commonly occur. As it appeared to be very difficult to find physiological differences between these organisms [10,11], the question arose what the difference in their functional status in the natural environment might be.

Competition studies on mixed cultures of *T. thioparus* and *T. pelophila* in thiosulfate-limited chemostats showed that at pH values above 7.5, *T. pelophila* outgrew *T. thioparus* at any thiosulfate concentration, whereas the reverse was true at pH values below 6.5 [7]. This thus means that at intermediate pH values the μ–s curves of both organisms should cross. And therefore under these conditions the results of competition depend on the thiosulfate concentration. A similar result was obtained when at pH 6.8 both organisms competed for growth-limiting iron (Fig. 3). In this case *T. pelophila* was shown to grow faster than *T. thioparus* at extremely low iron concentrations. The ecological significance of this finding is difficult to assess and is related to the question of whether iron limitation under natural conditions is feasible. Colorless sulfur bacteria thrive in the interface between aerobic and anaerobic conditions where both sulfide and oxygen are present [8]. In the higher region, at a relatively high redox potential (and high oxygen concentration), the available (ferric) iron concentration in the seawater may be very low [12]. On the other hand, at low redox potentials, where sulfide is present, the available iron concentration may be low due to the low value of the solubility product of ferrous sulfide. Thus it seems feasible that iron limitation under natural conditions may occur. Therefore, the ability of *T. pelophila* to grow faster than *T. thioparus* at low iron concentrations may be of ecological advantage and, at the same time, explain its higher sulfide tolerance.

In competition experiments with the chemostat one often finds that the organism growing faster at the lower concentration range of the limiting substrate is spiral-shaped. The surface-to-volume ratio of such organisms is generally higher than that of organisms growing faster at higher concentrations. Examples are shown in Table 1, referring to three couples of chemoorganotrophic and one of chemolithotrophic bacteria.

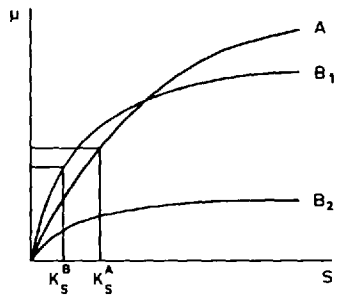

Fig. 4. Specific growth rate (μ) of organisms A, B_1, and B_2 as a function of the growth-limiting substrate (s). Surface/volume ratio $B_1 > B_2$; $k_s^{B1} = k_s^{B2}$.

The surface-to-volume ratio affects the maximal rate of growth, but not the k_s for growth. This is illustrated in Fig. 4, showing a hypothetical organism A competing with two organisms, B_1 and B_2. The k_s values of B_1 and B_2 are the same, but the surface-to-volume ratio of B_1 is greater than that of B_2. Even though a higher μ_{max} of B_1 with respect to B_2 does not help B_1 in competition with A at the higher substrate concentration range, it does give B_1 an advantage with respect to A at the lower substrate concentration range.

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