



WASHINGTON STATE  
UNIVERSITY



## INTERLIBRARY LOAN

Warning: this work may be protected by the copyright laws of the United States, Title 17, United States Code.

The WSU Libraries' goal is to provide excellent customer service. Let us know how we are doing by responding to this short survey:

[https://libraries.wsu.edu/access services survey](https://libraries.wsu.edu/access-services-survey)

Washington State University Pullman

Document Delivery



ILLiad TN: 1318243

**Journal Title:** Archives of microbiology  
**Volume:** 149  
**Issue:** 6  
**Month/Year:** 4 1988  
**Pages:** 509-514

**Article Author:** Oelze,

**Article Title:** Dependency of growth yield,  
maintenance and K s-values on the dissolved  
oxygen concentration in continuous cultures of  
Azotobacter vinelandii

**Imprint:** primo.exlibrisgroup.com-cross

**Date:** November 3, 2020

**Call #:** QK504 .A7 v.148-149 (1987/1988)

**Location:** owen

**Item #:**

Color Copies Requested? No

**\*\*ODYSSEY\*\***

**CUSTOMER HAS REQUESTED:**  
Electronic Delivery  
Electronic Delivery? Yes

Alexander Alleman (alexander.alleman)  
299 Clark Hall WSU  
Pullman, Wa 99163

Washington State University

NOV 03 2020

Library

# Dependency of growth yield, maintenance and $K_s$ -values on the dissolved oxygen concentration in continuous cultures of *Azotobacter vinelandii*

J. Kuhla and J. Oelze

Institut für Biologie II (Mikrobiologie), Universität Freiburg, Schänzlestrasse 1, D-7800 Freiburg, Federal Republic of Germany

**Abstract.** *Azotobacter vinelandii* was grown diazotrophically in sucrose-limited chemostat cultures at either 12, 48, 108, 144 or 192 µM dissolved oxygen. Steady state protein levels and growth yield coefficients ( $Y$ ) on sucrose increased with increasing dilution rate ( $D$ ). Specific rate of sucrose consumption ( $q$ ) increased in direct proportion to  $D$ . Maintenance coefficients ( $m$ ) extrapolated from plots of  $q$  versus  $D$ , as well as from plots of  $1/Y$  versus  $1/D$  exhibited a non-linear relationship to the dissolved oxygen concentration. Constant maximal theoretical growth yield coefficients ( $Y^G$ ) of 77.7 g cells per mol of sucrose consumed were extrapolated irrespective of differences in ambient oxygen concentration. For comparison, glucose-, as well as acetate-limited cultures were grown at 108 µM oxygen. Fairly identical  $m$ - and  $Y^G$ -values, when based on mol of substrate-carbon with glucose and sucrose grown cells, indicated that both substrates were used with the same efficiency. However, acetate-limited cultures showed significantly lower  $m$ - and, at comparable  $D$ , higher  $Y$ -values than cultures limited by either sucrose or glucose. Substrate concentrations ( $K_s$ ) required for half-maximal growth rates on sucrose were not constant, they increased when the ambient oxygen concentration was raised and, at a given oxygen concentration, when  $D$  was decreased. Since biomass levels varied in linear proportion to  $K_s$  these results are interpreted in terms of variable substrate uptake activity of the culture.

**Key words:** *Azotobacter vinelandii* – Dinitrogen fixation – Maintenance coefficients – Maximum growth yield coefficient  $K_s$ -values – Continuous culture

Through numerous investigations it is known that chemotrophic bacteria use the carbon and energy source not only for the production of biomass but also for so-called maintenance purposes (Pirt 1965). Recently, Tempest and Neijssel (1984) discussed a variety of physiological activities which do not directly lead to biomass accumulation and which, therefore, represent reactions consuming so-called maintenance energy. In order to quantify the specific rate of

substrate consumption fulfilling functions other than growth, Pirt (1965, 1975) developed the following equation:

$$q = \mu/Y^G + m,$$

where  $q$  is the specific rate of substrate consumption (mol substrate per g biomass per h),  $\mu$  ( $\text{h}^{-1}$ ) is the specific growth rate (= dilution rate,  $D$ , in chemostat cultures),  $m$  is the specific rate of substrate consumption required for maintenance purposes, and  $Y^G$  is the theoretical maximum growth yield coefficient (g biomass per mol substrate consumed) when the relative contribution of  $m$  to total  $q$  becomes negligibly low. Thus, plotting  $q$  versus  $\mu$  (or  $D$ ) yields a linear relationship with a slope equal to  $1/Y^G$  and an intersection on the ordinate representing  $m$ .

Alternatively, dividing the above equation by  $\mu$  provides a reciprocal relationship between  $Y$  and  $\mu$ :

$$1/Y = 1/Y^G + m/\mu.$$

In this relationship  $1/Y^G$  is equal to the intersection on the ordinate of a plot of  $1/Y$  versus  $1/\mu$  (or  $1/D$ ), while the slope equals  $m$ .

Members of the aerobic Azotobacters are well-known for their unusually high respiratory rates (Postgate 1982; Post et al. 1983) which develop under conditions of ammonium-limitation and stay constant at maximal rates when cells fix dinitrogen (Bühler et al. 1987a, b). Since high respiratory rates represent a highly uncoupled energy metabolism (Haddock and Jones 1977) dinitrogen fixing cells of Azotobacters should exhibit high maintenance requirements. In fact, comparing ammonium-assimilating and dinitrogen-fixing cultures of *Azotobacter chroococcum*, Dalton and Postgate (1969) observed an extremely increased maintenance rate with the latter culture. Nagai et al. (1971, 1972) extended these investigations with either oxygen- or glucose-limited chemostat cultures of *Azotobacter vinelandii*. Apart from corroborating a high maintenance rate, however, plots of specific rates of substrate consumption versus growth rate showed a negative slope which means negative  $Y^G$ . The authors explained this biologically irrelevant result by presuming steadily decreasing availability of oxygen when  $D$  was increased although the aeration rate was kept constant (Nagai and Aiba 1972). Consequently, results available, as yet, on the energetics of growth of Azotobacter are based on experiments performed under conditions of uncontrolled dissolved oxygen concentrations. Oxygen, however, is an important factor controlling the efficiency of substrate assimilation into biomass, as well as the coupling of energy metabolism. Therefore, we investigated mainte-

Offprint requests to: J. Oelze

**Abbreviations:**  $D$ , dilution rate;  $K_s$ , substrate concentration required for half maximal growth rate;  $m$ , maintenance coefficient;  $q$ , specific rate of substrate consumption;  $Y$ , growth yield coefficient;  $Y^G$ , maximum theoretical growth yield coefficient

nance, as well as growth yield coefficients in *A. vinelandii* growing at defined and constant oxygen concentrations. It will be shown that  $Y^G$ -values are positive and independent of different ambient oxygen concentrations while maintenance coefficients are related to the dissolved oxygen concentration. At a given oxygen concentration, however, both maintenance and yield coefficients depend on the carbon source.

## Materials and methods

*Azotobacter vinelandii*, strain OP (ATCC 13705), was grown under conditions of dinitrogen fixation at 30°C in an oxygen controlled carbon-limited continuous culture system (Post et al. 1982) at dilution rates between  $D = 0.05 \text{ h}^{-1}$  and  $D = 0.30 \text{ h}^{-1}$ . Dissolved oxygen concentrations between 12 and 192 µM were controlled with an autoclavable oxygen electrode (Ingold, Frankfurt, FRG) and kept constant with a proportional-integral operating valve (B. Braun, Melsungen, FRG). Growth-limiting substrates were either sucrose (0.3%), glucose (0.3%) or acetate (0.3%). For the determination of dry weight, cells were filtered through a 0.22 µm pore size filter with a filtration device (both Millipore, Bedford, USA) and dried at 100°C to constancy. Protein was measured according to Lowry et al. (1951). Total nitrogen contents of cells were determined with the micro-Kjeldahl method (Beloserski and Proskurjakow 1956). Substrate concentrations of sucrose, glucose or acetate in the culture supernatants were quantified with commercially available test combinations (Boehringer, Mannheim, FRG).

## Results

In order to study energetics of growth with chemostat cultures it is essential to exactly define and keep the growth-limiting conditions constant. Diazotrophic cultures of *Azotobacter vinelandii* employed throughout the present investigation were limited by the carbon- and energy-source. In addition dissolved oxygen concentrations were kept constant at defined levels. Limitation by the carbon/energy source was tested by increasing the concentration of the limiting substrate in the influent medium by a factor of five. This resulted in a five-fold increase of the steady state protein level ( $\bar{x}$ ) while the steady state substrate concentration ( $\bar{s}$ ) in the culture fluid, as well as the growth yield coefficient ( $Y$ ) remained constant. In addition, the composition of cells stayed constant as assumed on a constant ratio of  $0.70 \pm 0.04 \text{ g}$  of protein per g of dry weight of cells. However, total nitrogen contents of cells varied from  $17.4\% \pm 0.6\%$  of the protein content to  $19.4\% \pm 0.7\%$  when the dilution rate ( $D$ ) was increased from  $D = 0.05 \text{ h}^{-1}$  to  $D = 0.30 \text{ h}^{-1}$ . This may be explained by the fact that, with increasing growth rate, the RNA contents of cells increase (Khmel and Andreeva 1967). Because of the largely constant nitrogen, as well as protein contents on a dry weight basis, it could be ruled out that cells accumulated significant amounts of reserve material. This means that, in the following, protein can be used to estimate biomass formation.

When *A. vinelandii* was grown sucrose-limited at different oxygen concentrations different steady state protein levels were obtained depending, in inverse proportion, on the ambient oxygen concentration (Fig. 1). Protein levels, however, increased when  $D$ -values were increased up to

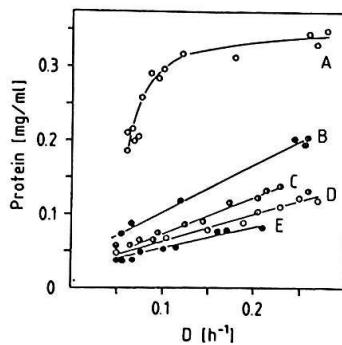


Fig. 1. Steady state protein levels of sucrose-limited chemostat cultures of *Azotobacter vinelandii* growing diazotrophically at 12 (○ A), 48 (□ B), 108 (● C), 144 (○ D) and 192 µM (● E) dissolved oxygen and various dilution rates ( $D$ ). The sucrose concentration of the inflowing medium was 3 g per l

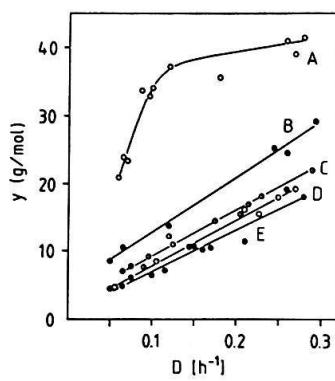


Fig. 2. Molar growth yield coefficients ( $Y$ ) on sucrose in chemostat cultures of *Azotobacter vinelandii* growing at different dilution rates ( $D$ ) and dissolved oxygen concentrations as described in Fig. 1.  $Y$ -values were calculated from protein levels and molar amounts of sucrose consumed

about  $D = 0.27 \text{ h}^{-1}$ . With cultures growing at 12 µM dissolved oxygen, this increase could be observed only up to  $D$  of about  $0.10 \text{ h}^{-1}$ , while at higher  $D$ -values protein levels approached constancy. Since essentially all of the substrate was used up by the organisms (Fig. 3a),  $Y$ -values exhibited a dependency on oxygen, as well as on  $D$  (Fig. 2) which was largely comparable to steady state biomass levels.

Oxygen dependent differences in steady state substrate concentrations ( $\bar{s}$ ), as depicted in Fig. 3a, require further consideration. According to the theory of continuous culture  $\bar{s}$  should depend entirely on the dilution rate,  $D$ . This follows from the following equation:

$$\bar{s} = K_s \frac{D}{\mu_{\max} - D},$$

where  $K_s$  represents the substrate concentration required to half-maximally saturate the maximal growth rate,  $\mu_{\max} = 0.34 \text{ h}^{-1}$ . Since  $\mu_{\max}$  did not exhibit different values when sucrose-limited cultures were grown at different oxygen concentrations, the equation infers variation of  $K_s$ . In fact, calculation of  $K_s$  revealed not only changes of the values as a response to different oxygen concentrations but also to variation of  $D$  (Fig. 3 b). Apparently, at higher  $D$ -values  $K_s$  approached constantly low levels. At a dissolved oxygen as low as 12 µM, however,  $K_s$  was essentially constant.

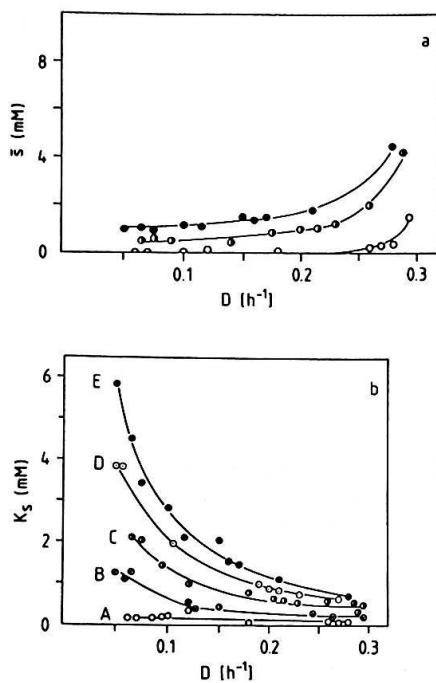


Fig. 3. a Steady state sucrose concentrations ( $S$ ) and b sucrose concentrations at which the cultures of *Azotobacter vinelandii* showed half maximum growth rate ( $K_s$ ) during growth at different oxygen concentrations (described in Fig. 1) and dilution rates ( $D$ )

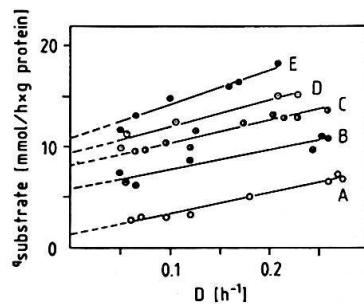


Fig. 4. Specific rates of sucrose consumption ( $q$ ) in chemostat cultures of *Azotobacter vinelandii* growing at various dissolved oxygen concentrations as described in Fig. 1

Contrasting the results of Nagai and Aiba (1972), rates of substrate consumption ( $q$ ) always increased linearly with  $D$  in spite of considerable differences in ambient oxygen concentrations (Fig. 4). But increased oxygen concentrations influenced  $q$ -values by leading to increased rates of substrate consumption at comparable dilution rates. In order to determine  $m$  and  $Y^G$  on sucrose the results were plotted according to the alternatives as described in the Introduction. Both methods differ in that plots of  $q$  versus  $D$  reveal deviations more readily from linearity while plots of  $1/Y$  versus  $1/D$  (Fig. 5) emphasize data as measured particularly at low  $D$ -values. Table 1 compiles the results of both calculations. Obviously, oxygen strongly increased the maintenance coefficient ( $m$ ) during growth on sucrose. Nevertheless,  $m$  did not increase linearly with increasing ambient oxygen concentration.  $Y^G$ -values on sucrose, however, were independent of the different oxygen concentrations employed (Fig. 5). The data of Table 2 reveal a mean value of  $Y^G = 54.4 \pm 3.3$  g of cell protein produced per mol

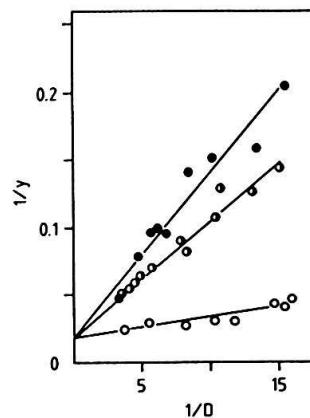


Fig. 5. Reciprocal plots of molar growth yield coefficients ( $Y$ ) versus dilution rate ( $D$ ). Depicted are the values obtained at 12 (○), 108 (●) and 192 μM (●) dissolved oxygen (see Fig. 2)

Table 1. Maintenance coefficients ( $m$ ) at various dissolved oxygen concentrations in sucrose (SUCR)-, glucose (GLC)- or acetate (ACE)-limited chemostat cultures of *Azotobacter vinelandii*. The  $m$ -values of column 1 were derived from plots of  $q$  versus  $D$ . Column 2 shows  $m$ -values derived from plots of  $1/D$  versus  $1/Y$

Carbon source	Oxygen conc. μM O <sub>2</sub>	Maintenance coefficient mmol <sub>substr</sub> /h·g		Maintenance coefficient mmol <sub>carbon</sub> /h·g	
		1	2	1	2
SUCR	12	1.2	1.4	14.4	16.8
	48	5.8		69.6	
	108	8.2	8.5	98.4	102.0
	144	9.2		110.4	
	192	10.5	12.5	126.0	150.0
GLC	108	19.6	17.5	117.6	105.0
ACE	108	15.5	46.6	31.0	93.2

Table 2. Maximum theoretical growth yield coefficients ( $Y^G$ , g protein or g dry weight per mol of substrate or per mol of substrate-carbon) of *Azotobacter vinelandii* grown in sucrose-limited continuous cultures at 12, 108 and 192 μM dissolved oxygen and either glucose- or acetate-limited cultures at 108 μM oxygen. The values were extrapolated from plots of  $1/Y$  versus  $1/D$

Carbon source	O <sub>2</sub> conc. μM	$Y^G_{\text{substr}}$ g/mol		$Y^G_{\text{carbon}}$ g/mol		Significance
		prot.	dry wt.	prot.	dry wt.	
SUCR	12	55.1	78.7	4.59	6.56	$r = 0.912 n = 11$
SUCR	108	50.9	72.7	4.24	6.06	$r = 0.983 n = 12$
SUCR	192	57.3	81.6	4.78	6.83	$r = 0.980 n = 8$
GLC	108	28.2	40.3	4.70	6.71	$r = 0.972 n = 13$
ACE	108	12.1	17.3	6.05	8.64	$r = 0.994 n = 9$

of sucrose assimilated. On the constant ratio of protein per dry weight (i.e. 0.70 g protein per g of cells) this means  $Y^G = 77.7$  g dry weight of cells per mol of sucrose consumed.

In order to evaluate possible effects of the carbon/energy source on the energetics of growth, additional experiments were performed with either glucose- or acetate-limited

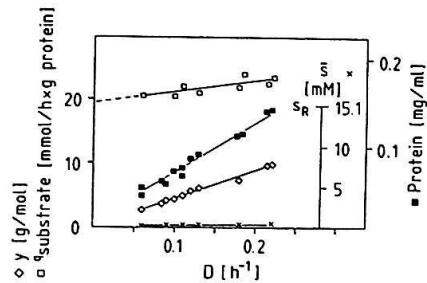


Fig. 6. Steady state protein levels (■); growth yield coefficients ( $y$ ), (◇); specific rates of substrate consumption ( $q$ ), (□) and steady state substrate concentrations ( $s$ ), (×) in a glucose-limited (0.3% chemostat culture of *Azotobacter vinelandii* growing diazotrophically at 108 µM dissolved O<sub>2</sub>

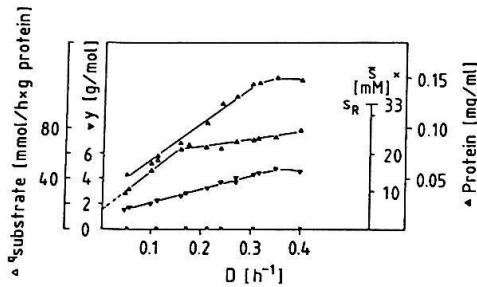


Fig. 7. Steady state protein levels (▲); growth yield coefficients ( $y$ ), (▼); specific rates of substrate consumption ( $q$ ), (△) and steady state substrate concentrations ( $s$ ), (×) in an acetate-limited (0.3%) chemostat culture of *Azotobacter vinelandii* growing diazotrophically at 108 µM dissolved O<sub>2</sub>

cultures growing at a concentration of 108 µM oxygen. With both substrates steady state protein levels, specific rates of substrate consumption, as well as growth yield coefficients increased with increasing growth rates (Figs. 6 and 7). Steady state glucose concentrations in the culture fluid were at least five-times less than concentrations of the substrate in sucrose-limited cultures while steady state acetate concentrations were below the sensitivity of the assay employed (< 10 µM).

$m$ - and  $Y^G$ -values on glucose and acetate, respectively, were determined as described above. For a better comparison of the data obtained with different carbon-sources the values compiled in Tables 1 and 2 are presented on a molar basis, as well as on the basis of substrate-carbon. As might be expected,  $m$ - and  $Y^G$ -values on glucose were half as high as on sucrose. When calculated on a carbon basis they were practically identical with cultures growing at identical oxygen concentration.

Different results were obtained, however, when acetate was the sole carbon/energy source at 108 µM dissolved oxygen. Although  $Y$ -values increased linearly with increasing growth rate, there were two ranges of linear relationship between  $q$ -values and  $D$  (Fig. 7). The data of Table 1 reveal that this led to different  $m$ -values when extrapolated from plots of either  $q$  versus  $D$  or  $1/Y$  versus  $1/D$ . Moreover,  $Y^G$  on acetate was significantly higher than could be expected on the corresponding data obtained with sucrose and glucose as substrates. These differences became more evident when maintenance and yield were calculated on a substrate-carbon basis (Table 1 and 2).

Table 3. The dependency on dissolved oxygen concentrations of dilution rates ( $D$ ) at which  $Y^G$  presumably becomes half maximal in diazotrophic continuous cultures of *Azotobacter vinelandii* limited by either sucrose, glucose or acetate

Carbon source	µM O <sub>2</sub> dissolved	Dilution rate h $^{-1}$
SUCR	12	0.0774
SUCR	108	0.4340
SUCR	192	0.7215
GLC	108	0.4937
ACE	108	0.5644

## Discussion

According to the theory of continuous chemostat cultures steady state biomass levels stay largely constant although the dilution rate ( $D$ ) may be varied within a relatively wide range. At very low  $D$ -values (compared to the maximal growth rate), however, biomass levels decrease because the rate of supply of the limiting substrate approaches the rate of substrate consumption fulfilling maintenance functions, i.e. functions other than biomass accumulation (Schultz and Gerhardt 1969). Concomitantly, growth yield coefficients ( $Y$ ) decrease. When *Azotobacter vinelandii* was grown diazotrophically in a sucrose-limited chemostat steady state biomass levels approached constancy only when the ambient oxygen concentration was as low as 12 µM and, even in this case, only when  $D$  was higher than 0.10 h $^{-1}$ . At all of the higher oxygen concentrations tested, biomass levels increased as  $D$  was increased. The slope of this increase, as well as the total levels of biomass became lower when the dissolved oxygen concentration was raised. Since  $Y$ -values exhibited a comparable behaviour, these results suggest that the maintenance coefficient ( $m$ ) became extraordinarily high so that it influenced the conversion of sucrose into biomass, except for the culture growing at 12 µM oxygen, even at the highest  $D$ -values used in this investigation. At still higher  $D$ -values the culture density began to decrease, indicating that limitations by the maximal growth rate  $\mu_{\max}$  on sucrose and glucose = 0.34 h $^{-1}$  and on acetate between 0.45 and 0.50 h $^{-1}$  and, thus, limitations by the critical dilution rate prevented further increases of biomass levels and  $Y$ -values up to a plateau region. In other words, increasing  $m$ -values by increasing ambient oxygen concentrations resulted in an expansion of the range of proportionality between  $Y$ -values, as well as biomass levels and  $D$  toward the highest possible  $D$ -values.

From the experimental data of this investigation, the theoretical maximal  $Y$ -values ( $Y^G$ ), as well as the dilution rates required for half-maximal  $Y^G$ -values, were extrapolated. Table 3 compiles the latter results obtained with sucrose-limited cultures as compared with cultures limited by either glucose or acetate. Through this it becomes evident that at higher oxygen concentrations the carbohydrate-limited cultures cannot even approach 1/2  $Y^G$  because of the relatively low  $\mu_{\max}$ . Although these values are theoretical, they are important in order to quantify the influence of oxygen on  $Y$ , i.e. on the slopes of the straight lines in Fig. 2. Moreover,  $Y^G$ -values revealed that, independent of ambient oxygen, 26.2% of the totally consumed carbon from carbohydrates were theoretically convertible into biomass. The extremely inefficient carbon assimilation becomes more

evident when it is remembered that ammonium-assimilating cultures of *Klebsiella aerogenes* convert about 67% of the carbon source into biomass (Tempest and Neijssel 1984). Since extrapolation of  $Y^G$  eliminates maintenance functions including uncoupled respiration, this presumes that low  $Y^G$ -values represent not only the provision of reducing equivalents and metabolic energy for dinitrogen fixation but also energy required for the cyclic retention of ammonium (Kleiner 1985), as well as for the formation of high levels of guanosine-5'-diphosphate-3'-diphosphate (ppGpp) characteristic of diazotrophic organisms (Kleiner and Phillips 1981).

High maintenance requirements of *A. vinelandii* were interpreted to represent a high degree of energetically uncoupled respiration and, thus, uncoupled growth (Nagai and Aiba 1972; Stouthamer 1979). If, as proposed, uncoupled respiration protects nitrogenase from irreversible oxygen damage,  $m$ -values and ambient oxygen concentration should be presumed to vary directly in constant proportion. Quantitation of  $m$ -values at defined oxygen concentrations showed a 8.5-fold increase while the ambient oxygen concentration increased by a factor of 16. But the proportionality between both was linear only at the lower oxygen concentrations while at higher concentrations  $m$ -values decreased from linearity. This suggests that, at higher oxygen concentrations, a lower proportion of the totally dissolved oxygen can be removed by uncoupled respiration than at low ambient oxygen. This agrees with measurements of total oxygen consumption by the cells (Post et al. 1983) and suggests that respiratory protection cannot be equally effective in protecting nitrogenase at different oxygen concentrations.

Lack of a linear proportionality between  $m$ -values and the presumed respiratory protection of nitrogenase is also indicated by the results obtained with acetate-limited cultures growing at 108 µM dissolved oxygen. In this latter case,  $m$ -values (calculated per substrate-carbon) were lower than in the case of carbohydrate-limited cultures growing at 108 µM dissolved oxygen, as well. Nevertheless, at comparable  $D$ -values steady state protein levels, as well as  $Y$ -values were higher with acetate-limited cultures. Since the nitrogen contents of protein stayed constant (Kuhla et al. 1985) the latter results imply increased dinitrogen fixation.

Sub-optimal biomass levels, because of extremely high  $m$ -values, provide a basis to explain the unexpected variation of  $K_s$ -values as response to variation not only of dissolved oxygen concentrations but also of  $D$ . The latter dependency of  $K_s$  on  $D$  at constant ambient oxygen excludes the possibility that variations of  $K_s$  resulted only from oxygen dependent changes in the aerobic metabolism of sucrose. It cannot be excluded, however, that, at constant  $D$ , variation of the oxygen concentration influenced the metabolic pattern of *A. vinelandii* and thus  $K_s$ .  $K_s$ , the substrate concentration allowing the rate of substrate-limited growth to become half-maximal, is derived from the Michaelis-Menten equation and represents the Michaelis constant ( $K_i$ ) for substrate consumption, assumed to be limited primarily by transport functions. As pointed out by Button (1983, 1985),  $K_s$  may vary at constant  $K_i$  when the transport activity of the entire culture varies. In the present case, sub-optimal biomass levels as a result of both ambient oxygen as well as  $D$  may be assumed to decrease the rate of sucrose consumption by the culture, thus increasing the steady state substrate level, ( $\bar{s}$ ), and consequently  $K_s$ . If this assumption is correct  $K_s$

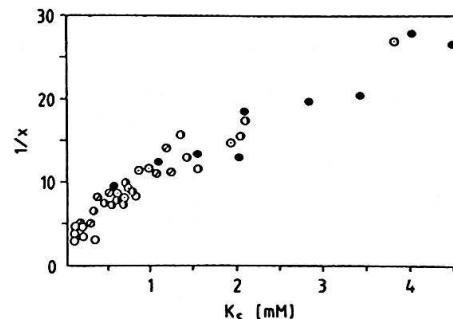


Fig. 8. Semireciprocal plot of steady state protein levels of sucrose-limited (0.3%) chemostat cultures of *Azotobacter vinelandii* versus steady state substrate concentrations at which the growth rate was half-maximal ( $K_s$ ). The organisms were grown diazotrophically at 12 (○), 48 (⊖), 108 (●), 144 (○) and 192 (●) µM dissolved oxygen (see Fig. 1)

should vary in inverse proportion to steady state biomass levels. The results depicted in Fig. 8 demonstrate not only that this is obviously true but also that, because of the essentially linear proportionality at  $K_s > 0.5$  mM, the activity of sucrose consumption per cell protein remained largely constant. At lower  $K_s$ -values, however a decrease from linearity appears possible.

**Acknowledgements.** We wish to thank Ch. Hoffmann for improving the English. This investigation was financially supported by the Deutsche Forschungsgemeinschaft grant Oe 55/11-6.

## References

- Beloserski AN, Proskurjakow L (1956) Praktikum der Biochemie der Pflanzen. Deutscher Verlag der Wissenschaften, Berlin
- Bühler T, Monter U, Sann R, Kuhla J, Dingler C, Oelze J (1987a) Control of respiration and growth yield in ammonium-assimilating cultures of *Azotobacter vinelandii*. Arch Microbiol 148:242–246
- Bühler T, Sann R, Monter U, Dingler C, Kuhla J, Oelze J (1987b) Control of dinitrogen fixation in ammonium-assimilating cultures of *Azotobacter vinelandii*. Arch Microbiol 148:247–251
- Button DK (1983) Differences between the kinetics of nutrient uptake by micro-organisms, growth and enzyme kinetics. Trends Biochem Sci 8:121–124
- Button DK (1985) Kinetics of nutrient-limited transport and microbial growth. Microbiol Rev 49:270–297
- Dalton H, Postgate JR (1969) Growth and physiology of *Azotobacter chroococcum* in continuous culture. J Gen Microbiol 56:307–319
- Haddock BA, Jones CW (1977) Bacterial respiration. Bacteriol Rev 41:47–99
- Khmel IA, Andreeva NB (1967) Physiological and biochemical factors governing growth of *Azotobacter vinelandii* in continuous cultivation on media with ammonium nitrogen. Mikrobiologiya 36:438–445
- Kleiner D (1985) Energy expenditure for cyclic retention of  $\text{NH}_3 \text{NH}_4^+$  during  $\text{N}_2$  fixation by *Klebsiella pneumoniae*. FEBS Lett 187:237–239
- Kleiner D, Phillips S (1981) Relative levels of ppGpp in some  $\text{N}_2$  fixing bacteria during repression and derepression of nitrogenase. Arch Microbiol 128:341–342
- Kuhla J, Dingler Ch, Oelze J (1985) Production of extracellular nitrogen-containing components by *Azotobacter vinelandii* fixing dinitrogen in oxygen-controlled continuous culture. Arch Microbiol 141:297–302

- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. *J Biol Chem* 193:265–276
- Nagai S, Aiba (1972) Reassessment of maintenance and energy uncoupling in the growth of *Azotobacter vinelandii*. *J Gen Microbiol* 73:531–538
- Nagai S, Nishizawa Y, Onodera M, Aiba S (1971) Effects of dissolved oxygen on growth yield and aldolase activity in chemostat culture of *Azotobacter vinelandii*. *J Gen Microbiol* 66:197–203
- Pirt SJ (1965) The maintenance energy of bacteria in growing cultures. *Proc Roy Soc [Lond], Ser B* 163:224–231
- Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford
- Post E, Golecki JR, Oelze J (1982) Morphological and ultrastructural variations in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. *Arch Microbiol* 133:75–83
- Post E, Kleiner D, Oelze J (1983) Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. *Arch Microbiol* 134:68–72
- Postgate JR (1982) The fundamentals of nitrogen fixation. University Press, Cambridge
- Schultz JS, Gerhardt P (1969) Dialysis culture of microorganisms: design, theory, results. *Bacteriol Rev* 33:1–47
- Stouthamer AH (1979) The search for correlation between theoretical and experimental growth yields. In: Quayle JR (ed) *Int Rev Biochem Microbial Biochemistry*, vol 21. University Park Press, Baltimore, pp 1–47
- Tempest DW, Neijssel OM (1984) The status of  $Y_{ATP}$  and maintenance energy as biological interpretable phenomena. *Annu Rev Microbiol* 38:459–486

Received September 25, 1987/Accepted November 13, 1987

## Base composition of DNA from symbiotic dinoflagellates: a tool for phylogenetic classification

Rudolf J. Blank<sup>1</sup>, Volker A. R. Huss<sup>1</sup>, and Walter Kersten<sup>2</sup>

<sup>1</sup> Institut für Botanik und Pharmazeutische Biologie, Universität Erlangen-Nürnberg, Staudtstrasse 5, D-8520 Erlangen, Federal Republic of Germany

<sup>2</sup> Institut für Biochemie der Medizinischen Fakultät, Universität Erlangen-Nürnberg, Fahrstrasse 17, D-8520 Erlangen, Federal Republic of Germany

**Abstract.** DNA of eight endosymbiotic dinoflagellates (zooxanthellae) from seven different host species has been analyzed as to its thermal characteristics and base composition by means of spectrophotometry and high performance liquid chromatography. All algae under investigation contain both methylcytosine and hydroxymethyluracil in addition to the bases typical of nuclear DNA. As a result, melting temperatures are decreased, suggesting lower contents of guanine plus cytosine than actually present. True percentages of guanine plus cytosine plus methylcytosine range from about 43 to 54 mol%. They are unique for the symbionts from different hosts, indicating phylogenetic separation of the taxa compared within the genus *Symbiodinium*.

**Key words:** Dinoflagellates – DNA composition – Hydroxymethyluracil – Methylcytosine – Speciation – *Symbiodinium* – Symbiosis – Zooxanthellae

Two morphologically distinct forms of dinoflagellates are currently known among eukaryotic endosymbionts in the marine environment. The group of amphidinioid zooxanthellae is composed of several species of the genus *Amphidinium* (Blank and Trench 1986; Trench and Blank 1987), while the gymnodinioid type was for a long time regarded as representing a single pandemic species of symbiotic dinoflagellate (cf. Freudenthal 1962; Taylor 1974), including varieties (cf. Taylor 1984) or nomina nuda (Holland and Carré 1974; Duclaux 1977). It has now been established that gymnodinioid symbionts are also heterogeneous, and that their species belong to the genus *Gymnodinium*.

*Offprint requests to:* R. J. Blank

**Abbreviations:** dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; dT, deoxythymidine; m5dC, 5-methyldeoxycytidine; hmdU, 5-hydroxymethyldeoxyuridine; rC, ribocytidine; Br8G, bromine-8-guanosine; A, adenine; C, cytosine; G, guanine; T, thymine; m5C, 5-methylcytosine; hmU, 5-hydroxymethyluracil; G + C, guanine plus cytosine plus 5-methylcytosine; HPLC, high performance liquid chromatography;  $T_m$ , temperature at the midpoint of hyperchromic shift; CTAB, N-cetyl-N,N,N-trimethylammonium bromide; EDTA, ethylenediamine-tetraacetic acid, disodium salt; TRIS, tris-(hydroxymethyl)-aminomethane; 1×SSC, standard saline citrate (0.15 M NaCl + 0.015 M trisodium citrate, pH 7.0)

*nium* (Spero 1987) as well as to the taxon *Symbiodinium* (Trench and Blank 1987).

*Symbiodinium* has been recorded free-living outside its hosts only twice (Loeblich and Sherley 1979; Taylor 1983). Studies were performed *in situ*, or on cultured symbionts *in vitro*. However, isolated zooxanthellae grow very slowly. It is difficult to bring them into culture, and even harder to bring them into mass culture. Therefore, it is not surprising that little emphasis has been placed on studying their DNA composition. To our knowledge, work including symbiotic dinoflagellates for DNA analyses is very rare (Franker 1970; Rae 1976). Data obtained from these studies have never served as arguments for phylogenetic discussions. Thus, even now the bulk of descriptions of symbiotic dinoflagellates is based on their morphology, with some more sophisticated techniques like behavioral, biochemical and physiological characterizations and karyotyping in tandem with three-dimensional reconstructions employed for the recognition of different species within the genus *Symbiodinium* (for review, see Blank and Trench 1985a,b; Trench and Blank 1987).

Besides the need for identifying zooxanthellae from different hosts as to their G+C contents, DNA of dinoflagellates in general bears interesting evolutionary aspects due to the occurrence of partially high amounts of methylated bases like methyladenine, methylcytosine and especially hydroxymethyluracil, substituting the bases customarily present in eukaryotes (cf. Rae and Steele 1978). We have therefore analyzed the DNA base composition of gymnodinioid zooxanthellae from seven different host species of five different orders of marine invertebrates. In order to meet representative selection of symbionts, we studied *Symbiodinium*-like dinoflagellates from a jellyfish, sea anemones, a zoanthid, and a stony coral, where the algae occur as intracellular cytosymbionts each enclosed within a single host vacuole in nature, as well as giant clams harboring intercellular endosymbionts in their haemal sinuses. Also, care was taken for choosing worldwide distribution of the hosts from which the symbionts had been originally isolated, using algae of invertebrates collected from Caribbean waters as well as from the Indopacific, with one identical host species originating from Palau and the Eniwetok atoll.

Our aim was to resolve three questions: (1) would different G+C compositions be uncovered in the DNA of zooxanthellae from different hosts; (2) are modified bases substituting for the canonical bases and to what extent; and