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Measurement of Growth at Very Low Rates ($\mu \geq 0$), an Approach To Study the Energy Requirement for the Survival of *Alcaligenes eutrophus* JMP 134

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Alcaligenes eutrophus **JMP 134 was grown in a recycling-mode fermenter with 100% biomass retention on 2,4-dichlorophenoxyacetic acid (2,4-D), phenol, and fructose. The growth pattern obtained given a constant supply of substrates exhibited three phases of linear growth on all three substrates. The transition from phase 1 to phase 2, considered to correspond to the onset of stringent (growth) control as indicated by a significant increase in guanosine 5′-bisphosphate 3′-bisphosphate (ppGpp), took place at 0.016 h^{-1} with 2,4-D and at about 0.02 h^{-1} with phenol and fructose. In the final phase, phase 4, which was achieved after the growth rate on the respective substrates fell below 0.003 to 0.001 h^{-1}, a constant level of biomass was obtained irrespective of further feeding of substrate at the same rate. The yield coefficients decreased by 70 to 80% from phase 1 to phase 3 and were 0 in phase 4. The stationary substrate concentrations s_{min} in phase 4, calculated from the kinetic constants of the strain, were 1.23, 0.34, and 0.23 μM for 2,4-D, phenol, and fructose, respectively. These figures characterize the minimum stationary substrate concentrations required in a dynamic system to keep *A. eutrophus* alive. This is caused by a substrate flux which enables growth at a rate ≥0 due to the provision of energy to an extent at least satisfying maintenance requirements. According to the constant feed rates of the substrates and the final and stable biomass concentrations, this maintenance energy amounts to 14.4, 4.0, and 2.4 μmol of ATP · mg of dry $mass^{-1}$ h^{-1} for 2,4-D, phenol, and fructose, respectively, after correction for the fraction of living cells. The increased energy expenditure in the case of 2,4-D is discussed with respect to uncoupling.**

Natural biotopes such as aquifers or (deeper) soil levels are characterized by physical and chemical factors which constitute extreme environments for microbial life. These biotopes represent oligotrophic conditions, which apply also to sites contaminated with organics of extremely low water solubility, i.e., very low concentration. For (chemoorgano-) heterotrophic microorganisms a major factor is the availability of carbon substrates. Low concentrations generally result in low growth rates. How microorganisms cope with such a situation and whether and how they are able to survive at extremely low concentrations is a question of the availability of energy, as the rate of energy flow results in the multiplication, maintenance, or ultimately, the death of cells. This is reflected by the kinetic characteristics of growth, which, from experimental experience at low and very low substrate concentrations, apparently deviate from the classical Monod pattern and the assumption of a growth rate-independent maintenance energy requirement as suggested by Pirt (21). Instead, rate-dependent terms are required to describe growth under such conditions according to models outlined elsewhere (5, 22, 28, 29). This behavior is attributed in part to regulatory mechanisms such as stringent (growth) control (3, 7, 30). Thus, attention should be paid to the gain and use of energy under conditions of extremely limiting substrate concentrations as reflected by the growth characteristics. Moreover, as pollutants are often toxic and toxicity may interfere with the energetic state of the cell (6, 11, 15, 18), this characteristic of substrates should be considered as a factor additionally modifying the energy budget actually available.

In order to gain insight into this topic with environmental implications, experiments were conducted with *Alcaligenes eutrophus* JMP 134 grown on 2,4-dichlorophenoxyacetic acid (2,4-D) and phenol, which are both toxic, as indicated by their inhibition kinetics (13, 20), and, for comparison, on fructose. The questions considered were (i) how much energy is required to maintain a given population of this strain alive, (ii) in which manner substrates known to become toxic influence this requirement, and (iii) which minimum stationary substrate concentration is necessary to enable growth at a rate of ≥0.

MATERIALS AND METHODS

Organism. *A. eutrophus* JMP 134 (8) was kindly provided by H. J. Knackmuss (University of Stuttgart, Stuttgart, Germany). The strain was stored on agar slants on a minimal medium containing 1 mM 2,4-D or 2 mM phenol; to initiate growth on a liquid minimal medium, 0.5 mM 2,4-D or 2 mM phenol was supplied. For long-term deposit, the strain was stored in liquid nitrogen after growth on a minimal medium containing 0.5 mM 2,4-D.

Culture conditions. Cultivation was performed in an Infors (Einsbach, Germany) ISF 100 laboratory fermenter with a working volume of 1 liter. The fermenter was equipped with a Fresenius (St. Wendel, Germany) hollow-fiber ultrafiltration module (0.2 μm; Polysulfone SPS 9005-4) and a Monitek (Düsseldorf, Germany) turbidity measuring device (model 22/CT4.1) equipped with a flowthrough cell (light path, 3 cm), both operated in a bypass. The cell suspension from the fermenter was continuously pumped through this bypass at a rate of 1 liter · min^{-1}. Growth with biomass recycling was induced by supplying the nutrient-substrate solution at a constant rate by an SSP-20 peristaltic pump (former German Academy of Sciences, Berlin), which maintains high flow constancy. The working volume of the system was kept constant by a conductometric sensor operating a second pump, which removed cell-free liquid via the hollow-fiber module (internal phase) at a rate equal to the feeding rate. Bacterial cells did not penetrate this membrane, and the biomass was retained completely within the fermenter. The rates of the supply of growth medium and the removal of culture broth were monitored via a personal computer recording data from a

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TABLE 1. Distribution of carbon during chemostatic growth of *A. eutrophus* JMP 134 on 2,4-D and phenol

Substrate (growth rate [h^{-1}])	Carbon content (%)			
	Biomass	Carbon dioxide	Supernatant[a]	Total
2,4-D (0.065)	20.42	81.07	2.23	103.72
Phenol (0.06)	43.28	55.12	3.06	101.46

[a] Cell-free culture broth.

balance carrying the reservoir flask and the operation frequence of the second pump, respectively. Any deviations from stable hydrodynamic conditions, i.e., a constancy of the liquid level in the cultivation vessel by equilibrating the inflow (growth medium) and outflow (cell-free culture broth) of liquid, could thus be observed, and the mass flow balances could be rectified if necessary. Growth in liquid culture took place in a minimal medium which was composed of (in milligrams per liter) NH_4Cl, 760; KH_2PO_4, 680; $MgSO_4 \cdot 7H_2O$, 71.2; $CaCl_2 \cdot 6H_2O$, 5.5; $FeSO_4 \cdot 7H_2O$, 4.98; $CuSO_4 \cdot 5H_2O$, 0.785; $MnSO_4 \cdot 4H_2O$, 0.81; $ZnSO_4 \cdot 7H_2O$, 0.44; and $Na_2MoO_4 \cdot 4H_2O$, 0.25 (14). Fructose (5.5 mM), phenol (8.0 mM), and 2,4-D (10.0 mM, supplied as sodium salt) served as the carbon and energy source. The pH was held constant at 6.8 by titration with either 0.8 M NaOH or 0.8 M HCl. Growth temperature was 30°C; the dissolved-oxygen concentration in the fermentation broth ranged from 50 to 80% of air saturation as determined by an Ingold oxygen electrode.

Growth parameters. The kinetic parameters (μ_{max} and k_s) of growth on the various substrates were derived from continuous cultivation experiments (carbon substrate-limited chemostat). The stationary substrate concentrations were obtained after steady states were attained at specific dilution rates by rapidly separating biomass from the culture broth with a Falcon 7103 ultrafiltration device (0.22 μm) (Becton Dickinson, Paramus, N.J.). So, suspension from the fermenter was sampled by discharging five times the dead volume of the sample port within 5 s and then obtaining about 2-ml portions of cell-free filtrates from the fermenter's interior in a further 1 to 2 s without disrupting the liquid stream. Time constants of the sampling procedure allowed a fractional volume of suspension from the fermenter to be separated from biomass within 1 s (13). The stationary substrate concentration was properly fixed by this procedure and could significantly be measured down to 0.2 $mg \cdot liter^{-1}$ by the analytical methods. Kinetic constants were derived from these data by linear regression analysis of Lineweaver-Burk plots.

The respiration rate was determined in a Cyclobios Paar (Innsbruck, Austria) Oxygraph respirometer. Washed cells from the exponential growth phase cultivated on the respective substrates were resuspended in the growth medium, and the respiration rate was determined in the same medium (2 ml) saturated with air at 30°C. The respiration rate was derived from the initial rates of oxygen consumption after the substrate had been added in 50-μl quantities.

Analyses. The biomass concentration was measured off-line by optical density (OD) at 700 nm after appropriate dilution and by dry-mass determination (quadruplicate of cell pellets from 3 ml, 5 h at 105°C). To avoid disturbing growth, only two samples were withdrawn per day: dry mass and OD were determined from an aliquot of about 20 ml, and a second sample of about 5 ml was taken for a further OD measurement. In order to permanently follow up growth without taking samples, the OD signal monitored on-line was used as a trend indication. Dry-mass values were used for calculating the yield coefficients. The OD figures correlated to dry mass by an almost constant factor of 0.55 and were used for graphic presentation of the biomass concentration. To quantitate the fraction of active biomass, cell counts were determined. The total cell number was obtained by using a Multisizer II (Coulter Electronics Ltd., Krefeld, Germany) with a capillary aperture of 20 μm. Suspensions were diluted to 0.5×10^6 to 1×10^6 cells $\cdot ml^{-1}$, reducing the value of coincidence to 2 to 3%. Viable cell number was determined by plate counts on a complex medium (pH 7.0; 1 day at 30°C) containing 3% (wt/vol) each yeast extract and peptone and 10 mM fructose. The suspension was diluted to yield 100 to 300 CFU per plate. The cell numbers were derived from triplicates; standard deviation was less than 5% for both the total cell number and the viable cell number.

The concentration of ppGpp was determined by high-pressure liquid chromatography (HPLC) after metabolism was stopped and intracellular nucleotides were extracted by an alkaline procedure (17); ppGpp as a standard was kindly provided by R. Wagner (University of Düsseldorf, Düsseldorf, Germany). Organic carbon from the growth medium and that from the cell-free supernatant of the culture broth were measured by a liquiTOC analyzer (Voss-Heraeus). The carbon content of biomass was obtained from a CHN-1000 analyzer (Leco) after washed cells were dried overnight at 105°C. Phenol and 2,4-D were measured by HPLC (19). Fructose was measured enzymatically via hexokinase, phosphoglucoisomerase, and glucose 6-phosphate dehydrogenase (4), by increasing the sample portion up to 10-fold.

RESULTS

Growth characteristics at very low rates. Cultivation was performed by a recycling mode technique with biomass retention (1, 2). This method is suitable for establishing and maintaining growth at very low rates down to 0, enabling analysis of its physiological and biochemical background. For this, *A. eutrophus* JMP 134 was chemostatically grown on 2,4-D, phenol, and fructose. The distribution of substrate carbon into the different fractions at steady-state conditions is shown in Table 1. After attaining steady-state conditions the culture was shifted to a regimen with 100% biomass retention by further feeding substrate. This resulted in an increase in biomass with profiles as indicated in Fig. 1. In all cases, two phases (phase 2 and 3) of linear biomass increase and one phase (phase 4) of a constant biomass level were observed. The initial phase (phase 1) was rather short, as the dilution rates during chemostatic precultivation were only about 0.06 h^{-1}. The transition between the individual phases appeared rather abruptly. The changeover to phase 2 was accompanied by an increase in the intracellular concentration of the alarmone ppGpp with all three substrates. These figures rose by a factor of 3 to 8 and remained at this high level up to stationary growth (Fig. 2).

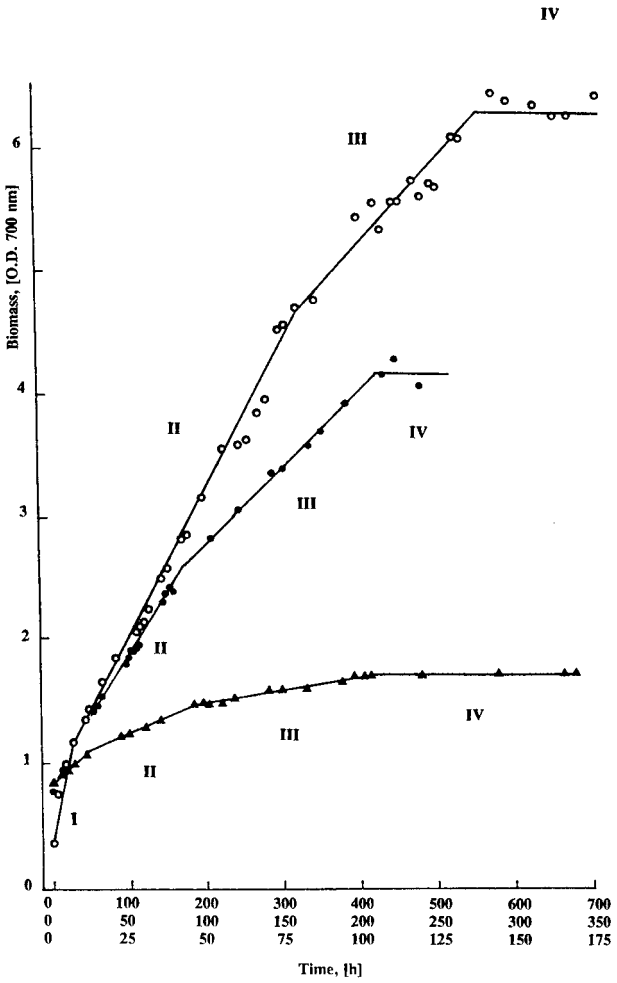

FIG. 1. Continuous cultivation on various substrates of *A. eutrophus* JMP 134 with 100% biomass retention. Substrates were fed at constant rates of 278 μmol of fructose per h (○; scale, 0 to 700 h), 430 μmol of phenol per h (●; scale, 0 to 350 h), and 755 μmol of 2,4-D per h (▲; scale, 0 to 175 h).

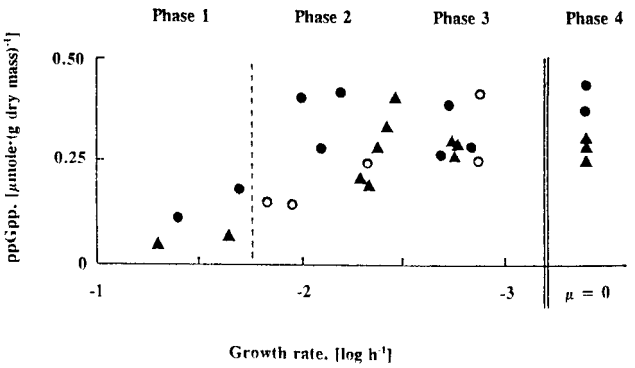

FIG. 2. Concentration of ppGpp during growth of *A. eutrophus* JMP 134 on various substrates. ppGpp was measured for cultures grown in a recycling-mode regimen at respective growth rates. A 9.5-ml sample of suspension was withdrawn from the fermenter within 5 s and put into ice-cold glass tubes containing 0.5 ml of 1 M KOH. ppGpp was extracted and measured as indicated elsewhere (17). ▲, 2,4-D; ●, phenol; ○, fructose.

The substrate provision rates (SPR) were not changed but kept constant throughout the whole experiment; consequently, the specific growth rates decreased and lastly attained 0. A highly constant SPR and stable hydrodynamic conditions are prerequisites to obtaining significant growth parameters from such experiments. This could be verified in the present case by monitoring the fluid streams; deviations were found to be less than 2% throughout the individual experiments and were not included in the calculations. The stoichiometric and kinetic parameters obtained from these cultivation experiments are summarized in Table 2. The specific rates in a given phase of linear growth were calculated according to the method of Van Verseveld et al. (28) as $\mu = \mathrm{SPR} \cdot Y^i/(X_0{}^i + t \cdot \mathrm{SPR} \cdot Y^i) = \mathrm{SPR} \cdot Y^i/X_t$, where μ is the specific growth rate (hour^{-1}), SPR is in $\mathrm{moles} \cdot \mathrm{liter}^{-1} \cdot \mathrm{hour}^{-1}$, Y^i is the growth yield coefficient (in grams of dry mass $\cdot$ mole^{-1}) in the respective growth phases (Table 2), and $X_0{}^i$ and X_t are the total biomass concentrations at the onset of the i^{th} growth phase ($X_0{}^i = X_0 + \Sigma t \cdot \mathrm{SPR} \cdot Y^{i-1}$ and at time t of a given growth phase ($X_t = X_0{}^i + t \cdot \mathrm{SPR} \cdot Y^i$), respectively; X_0 is the inoculum concentration (in grams of dry mass $\cdot$ liter^{-1}). Discontinuities in the growth rates which are obvious in the transition between the various phases resulted from the significant decrease of the yield coefficient (Table 2).

In order to estimate the fraction of active biomass in phase 4, viable cell counts were determined and correlated to the total cell number. According to this, this fraction was found to amount to about 75% with both fructose and phenol and to about 60% with 2,4-D under conditions of stationary growth. (The viable counts obtained from cell suspensions of the other growth phases revealed a fraction of active biomass of about 100% with fructose, 85% with phenol, and 60% with 2,4-D.) These ratios were used to verify the maintenance energy requirements in phase 4.

Energetics of growth and maintenance. The amount of energy required for growth and maintenance can be derived from the substrate conversion characteristics. According to the yield coefficients (Table 2), the dissimilated part of substrate, S_d, increased in the growth phases, meaning that more energy becomes potentially available for maintenance. Carbon substrate was quantitatively used for maintenance in phase 4. The S_d portion can be equated to ATP quantities. The rate of its production was calculated as $S_d = (c_s - \mathrm{Y}^i \cdot c_x)/c_s$, $r_{\mathrm{ATP}} = \mathrm{SPR} \cdot S_d \cdot V_{\mathrm{ATP}}$, and $r_{\mathrm{ATP,sp}} = r_{\mathrm{ATP}}/X_t$, where c_s and c_x ($c_x = 0.47$) are the fractions of carbon in substrate and biomass, respectively, and V_{ATP} is the molar quantity of ATP gained from the complete oxidation of a substrate molecule at a given P/O quotient (P/O, energy-rich phosphate bonds formed per oxygen atom consumed) (15). The results obtained in this way are shown in Table 3. A P/O-quotient of 2, which was derived from the yield coefficients obtained during growth on phenol and fructose (Table 2) (16), was taken into account. The specific rates decreased in the various phases, reflecting the restricted energy available.

From the growth pattern in phase 4, i.e., zero growth, the maintenance coefficient, m, can be calculated as $m = (f_m \cdot \mathrm{SPR})/X_f$, where f_m is the fraction of substrate used for maintenance, which is 1 at stationary growth, and X_f is the stationary biomass concentration at phase 4. Lastly, this value must be corrected by taking into account the fraction of living cells (see above). The maintenance energy demands thus calculated are shown in Table 4. The results indicate a moderate increase per biomass unit with phenol in comparison with fructose and a high rise with 2,4-D.

Minimum substrate concentration. For kinetic reasons it was of importance to determine stationary substrate concentrations required at minimum to sustain growth at a rate of 0, i.e., to maintain cells alive. The maintenance energy requirements are connected to kinetic data via the rate of negative growth, b, with $b = m \cdot Y_{\mathrm{max}}$. This follows from an extended Monod model of growth incorporating biomass decay (for derivation and application see references 21 to 23 and 25 to 27). The determination of b enables the calculation of this minimum stationary substrate concentration: $s_{\mathrm{min}} = k_s \cdot b/(\mu_{\mathrm{max}} - b)$. These results are also shown in Table 4. Negative growth rate was highest with 2,4-D, as is the s_{min} value with this substrate.

The k_s values required for calculation of s_{min} were derived from continuous cultivation experiments with fructose and 2,4-D as indicated in Materials and Methods. Linear and one-

TABLE 2. Yield coefficients and rates in the different phases of growth of *A. eutrophus* JMP 134 during recycling-mode cultivation on various substrates[a]

Phase[b]	Fructose		Phenol		2,4-D	
	Y^i [0.5]	μ [0.44]	Y^i [0.75]	μ [0.28[c,d]]	Y^i [0.18]	μ [0.215[c]]
1	0.39	0.0198[e]	0.394	0.0194[e]	0.102	0.0159[e]
2	0.154	0.0138–0.0027	0.290	0.0142–0.0074	0.064	0.0099–0.0076
3	0.086	0.0015–0.0011	0.192	0.0049–0.0031	0.023	0.0027–0.00235

[a] Y^i values are given in grams of dry mass $\cdot$ gram of substrate^{-1}; μ values are rates of growth per hour. Maximum values are given in brackets.
[b] For phase 4, all Y^i and μ values were 0.
[c] Virtual maximum, because of inhibition kinetics.
[d] During expression of the *ortho* cleavage pathway.
[e] Minimum.

TABLE 3. Rates of energy production in the linear phases of growth[a]

Phase	Fructose		Phenol		2,4-D	
	r_{ATP} [2.98]	$r_{ATP,sp}$ [5.18]	r_{ATP} [4.64]	$r_{ATP,sp}$ [7.12]	r_{ATP} [10.96]	$r_{ATP,sp}$ [16.28]
1	3.92	3.33[b]	6.5	6.94[b]	12.12	11.95[b]
2	5.92	5.01–2.01	7.15	7.63–3.65	12.67	12.50–9.33
3	6.50	2.21–1.62	7.53	3.84–2.60	13.27	9.77–8.51
4[c]	7.23	1.80 (2.4)	8.60	2.97 (4.0)	13.60	8.64 (14.4)

[a] r_{ATP} values are given in micromoles · liter^{-1} · hour^{-1}; $r_{ATP,sp}$ values are given in micromoles · milligram of dry mass^{-1} · hour^{-1}. Maximum values are given in brackets.
[b] Minimum.
[c] Values for the fractions of living cells are given in parentheses.

phase double-reciprocal plots were obtained at substrate concentrations from the detection limit (about 0.2 mg · liter^{-1}) to about 10 mg of fructose and 70 mg of 2,4-D liter^{-1} (not shown), resulting in the data given in Table 4. In the case of phenol, this method caused problems, as at lower growth rates (extrapolated to an apparent μ_{max} of 0.28 h^{-1}) this substrate was assimilated almost exclusively via the *ortho* cleavage pathway. Up to a dilution rate of $D = 0.25$ h^{-1} residual phenol was below the detection limit of 0.2 mg · liter^{-1}. At around this rate the *meta* cleavage pathway was additionally switched on, giving rise to a different metabolism, in which a μ_{max} of 0.415 h^{-1} and a k_s of 59 μM were observed (13). This fails to describe the behavior by using the *ortho* cleavage pathway and thus growth at low residual substrate concentrations. Accordingly, this kinetic parameter was derived from respiration experiments. According to Harrison (9) the rationale is to assume a correlation between substrate-dependent oxygen demand and growth rate of cells freshly harvested from the exponential growth phase. A suspension of *A. eutrophus* JMP 134 which expressed the *ortho* cleavage pathway from which a k_s value of 6.9 μM was obtained was used (the k_s values determined by this method coincide closely with those of continuous cultivation experiments, as is apparent from the data with fructose and 2,4-D in Table 4).

DISCUSSION

The amount of energy required to maintain a microbial cell and, thus, a population of such cells alive should be a constant under given environmental conditions. However, different requirements were found when various substrates were utilized with *A. eutrophus* JMP 134. In the case of fructose, 2.4 μmol of ATP is required per g of biomass (dry mass) per h; this value is only 1.7 times higher for phenol but 6 times higher for 2,4-D (Table 3). These quantities were derived from calculations by taking into consideration energy gained from all the substrate carbon being not fixed into biomass; this should be valid, as carbon being liberated by oxidative decarboxylation via assimilatory routes is assumed to deliver respective energy equivalents (see reference 16 and references therein). According to these data, fructose and phenol behave more similarly in that the energy contents of these substrates are transduced to nearly comparable extents, reflecting the degree of coupling of the respiratory chain, which corresponds to a P/O quotient of about 2 (16). 2,4-D deviates from this pattern in that the net energy gain by the metabolism is essentially smaller. This cannot, however, be attributed to the expenditure of dechlorination and ether bond cleavage in this substrate (16). Rather, this is thought to reflect a confrontation of the organism with this highly toxic substrate, as is obvious from the growth yield on 2,4-D amounting to only 0.18 g of dry mass · g of substrate^{-1}. From a theoretical consideration, this would reflect an apparent P/O quotient of about 1 (16). The reason for this behavior is not clearly evident. Estimation of the carbon balance during growth on 2,4-D supported the high portion of substrate dissimilated. Carbon was almost exclusively distributed into biomass and carbon dioxide, and no essential products were formed (Table 1). One explanation may be seen in uncoupling effects during growth on 2,4-D. It is known from comparative toxicity assessments, referred to as uncoupling phenomena, that, because of an increase in hydrophobicity and a decrease in the pKa value as the chlorine content of phenolics rises (for 2,4-D, see reference 10), the uncoupling property is reinforced compared with phenol (6). This property, however, needs further study.

The zero growth rate underlying the pattern in phase 4 corresponds to a definite stationary substrate concentration (Table 3). The occurrence of such minimal (available) substrate concentrations applies to most natural biotopes, defined as oligotrophic, in any dynamic system (12). A situation rather similar to natural (oligotrophic) environments may be expected for anthropogenically influenced ecosystems, contaminated by xenobiotic compounds which are often characterized by very limited water solubilities, so that microorganisms are steadily supplied with a low but almost constant flow of substrate, caused either by solubilization/diffusion properties (e.g., in contaminated soils) or by a constant flow of water polluted in some way (as may be true of groundwater aquifers, biofilters, etc.). Microbial growth will be possible as long as the available substrate concentration (i.e., its supply rate) exceeds s_{min}. Model studies of such dynamic systems with regard to growth, maintenance, and decay have already been reported (23, 24).

The profile of biomass concentration in the recycling-mode cultivation regimen exhibits clearly distinct phases of linear growth. This means that, despite the increase of biomass within such a phase, the yield calculated per unit of substrate may even increase. Any reference to the cell's storage materials is useless for explaining this effect, as they are not a source of extra dry mass. Rather, this pattern may be caused by the occurrence of subpopulations with different growth rates, or, in extreme cases, by an increase in the portion of the population shifting to a dormant state (7). The latter was also considered by Pirt (22) in his extended model of maintenance requirements. The assumption of a dormant fraction has the advantage of easily explaining linear growth by making energy all the more available to the active fraction the larger the fraction of dormant cells becomes. The assignment of cells to a dormant fraction and a growing fraction is difficult, however. It was observed in the present experiments that the titer of living cells was reduced to 75 to 60% at stationary growth; this fraction was considered homogenous in activity in the present calculations (Table 3). (That the specific energy production rates

TABLE 4. Constants of growth of *A. eutrophus* JMP 134 on various substrates

Substrate	m (μmol · mg^{-1} h^{-1})	s_{min} (μM)	k_s (μM)	b (h^{-1})
Fructose	0.070 (0.090[a])	0.21	14.0 (17[b])	0.0068
Phenol	0.162 (0.220[a])	0.30	6.9[b]	0.0115
2,4-D	0.475 (0.790[a])	1.23	12.8 (5[b])	0.0189

[a] Corrected for the fraction of living cells (CFU).
[b] Derived from respiration data (see Materials and Methods).

increased after transition to a respective phase compared with the preceding one follows from the abrupt increase in substrate dissimilated. This was most pronounced with fructose and less evident with 2,4-D.) Four distinct linear growth phases were similarly observed in the conversion of 3-chlorobenzoate by *Pseudomonas* sp. strain B13 (25), although no reference to a fraction of living cells was made. The growth phases and the transitions between them can partly be explained by a physiological response to energy shortage. This is indicated by the concentration of ppGpp, which significantly increased as the growth rates fell below about 0.02 h^{-1} (Fig. 2). This is considered an indication of the onset of stringent (growth) control. Similar effects concerning the concentration pattern of ppGpp have been observed with *Escherichia coli* and *Paracoccus denitrificans* at such low growth rates (28). However, further regulatory mechanisms seem to be involved in stabilizing metabolism in order for cells to survive and grow, as follows from the multiphasic pattern of growth rate at this very low range.

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REFERENCES

1. **Arbige, M., and W. R. Chesbro.** 1982. Rel A and related loci are growth rate determinants for *Escherichia coli* in a recycling fermentor. J. Gen. Microbiol. **128:**693–703.
2. **Arbige, M., and W. R. Chesbro.** 1982. Very slow growth of *Bacillus polymyxa*: stringent response and maintenance energy. Arch. Microbiol. **132:**338–344.
3. **Baracchini, E., and H. Bremer.** 1988. Stringent and growth control of rRNA synthesis in *Escherichia coli* are both mediated by ppGpp. J. Biol. Chem. **263:**2597–2602.
4. **Bernt, E., and H. U. Bergmeyer.** 1970. D-Fructose, p. 1266. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen Analyse, vol. 2. Akademie-Verlag, Berlin.
5. **Beyeler, W., P. L. Rogers, and A. Fiechter.** 1984. A simple technique for the direct determination of maintenance energy coefficient: an example with *Zymomonas mobilis*. Appl. Microbiol. Biotechnol. **19:**277–280.
6. **Bryant, S. E., and T. W. Schultz.** 1994. Toxicological assessment of biotransformation products of pentachlorophenol: *Tetrahymena* population growth impairment. Arch. Environ. Contam. Toxicol. **26:**299–303.
7. **Chesbro, W. R., T. Evans, and R. Eifert.** 1979. Very slow growth of *Escherichia coli*. J. Bacteriol. **139:**625–638.
8. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145:**681–686.
9. **Harrison, D. E. F.** 1973. Studies on the affinity of methanol and methane-utilising bacteria for their carbon substrates. J. Appl. Bacteriol. **36:**301–308.
10. **Landolt, H., R. Börnstein.** 1960. Konstanten der elektrolytischen Dissoziation, p. 879. *In* K. H. Hellwege, A. M. Hellwege, K. Schäfer, and E. Lax (ed.), Zahlenwerte aus Physik, Chemie, Astronomie, Geophysik, Technik. 6. Auflage, vol. 7/II. Springer-Verlag, Berlin.
11. **Loffhagen, N., C. Härtig, and W. Babel.** 1995. The glucose-dehydrogenase-mediated energization of *Acinetobacter calcoaceticus* as a tool for evaluating its susceptibility to, and defence against, hazardous chemicals. Appl. Microbiol. Biotechnol. **42:**526–531.
12. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1–23. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
13. **Müller, R. H.** Unpublished data.
14. **Müller, R. H., and W. Babel.** 1993. Oxidative capacity determines growth rate with *Acetobacter methanolicus*. Acta Biotechnol. **13:**3–11.
15. **Müller, R. H., and W. Babel.** 1993. Dinitrophenol influences the rate and the yield of *Acetobacter methanolicus*. Acta Biotechnol. **13:**243–249.
16. **Müller, R. H., and W. Babel.** 1994. Phenol and its derivatives as heterotrophic substrates for microbial growth—an energetic comparison. Appl. Microbiol. Biotechnol. **42:**446–451.
17. **Müller, R. H., N. Loffhagen, and W. Babel.** Rapid extraction of (di)nucleotides from bacterial cells and determination by ion-pair reversed-phase HPLC. J. Microbiol. Methods, in press.
18. **Neijssel, O. M., R. W. J. Hommes, P. W. Postma, and D. W. Tempest.** 1989. Physiological significance and bioenergetic aspects of glucose dehydrogenase. Antonie Leeuwenhoek **56:**51–61.
19. **Oh, K. H., and O. H. Tuovinen.** 1990. Degradation of 2,4-dichlorophenoxyacetic acid by mixed cultures of bacteria. J. Ind. Microbiol. **6:**275–278.
20. **Pieper, H. P., W. Reinecke, K. H. Engesser, and H. J. Knackmuss.** 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. **150:**95–102.
21. **Pirt, S. J.** 1965. Maintenance energy of bacteria in growing cultures. Proc. Soc. Lond. Ser. B **163:**224–231.
22. **Pirt, S. J.** 1987. The energetics of microbes at slow growth rates: maintenance energies and dormant organisms. J. Ferment. Technol. **65:**173–177.
23. **Rittmann, B. E., and P. L. McCarty.** 1980. Model of steady-state-biofilm kinetics. Biotechnol. Bioeng. **22:**2343–2357.
24. **Rittmann, B. E., and P. L. McCarty.** 1980. Evaluation of steady-state-biofilm kinetics. Biotechnol. Bioeng. **22:**2359–2373.
25. **Tros, M. E., G. Schraa, and A. J. B. Zehnder.** 1994. Biodegradation of 3-chlorobenzoate at low concentrations: transformation kinetics and residual concentrations, p. 70–72. *In* IChem^{E} Second International Symposium on Environmental Biotechnology, Brighton, United Kingdom. Chameleon Press Ltd., London.
26. **Van der Kooij, D., J. P. Oranje, and W. A. M. Hijnen.** 1982. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. Appl. Environ. Microbiol. **44:**1086–1095.
27. **Van der Kooij, D., A. Visser, and W. A. M. Hijnen.** 1980. Growth of *Aeromonas hydrophila* at low concentrations of substrates added to tap water. Appl. Environ. Microbiol. **39:**1198–1204.
28. **Van Verseveld, H. W., W. R. Chesbro, M. Braster, and A. H. Stouthamer.** 1984. Eubacteria have 3 growth modes keyed to nutrient flow. Arch. Microbiol. **137:**176–184.
29. **Van Verseveld, H. W., J. A. De Hollander, J. Frankena, M. Braster, F. J. Leeuwerik, and A. H. Stouthamer.** 1986. Modeling of microbial substrate conversion, growth and product formation in a recycling fermentor. Antonie Leeuwenhoek **52:**325–342.
30. **Wagner, R.** 1994. The regulation of ribosomal RNA synthesis and bacterial cell growth. Arch. Microbiol. **161:**100–109.