Long-Term Changes in Chemostat Cultures of *Cytophaga johnsonae*

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Long-term studies with a gliding, heterotrophic bacterium, *Cytophaga johnsonae*, were conducted in a glucose-limited chemostat at a high and a low dilution rate. To test the stability of the steady state during long-term experiments the following parameters were monitored: optical density, glucose concentration, glucose uptake potential, ATP content of the cells, and plate counts on two different agar media. Biomass remained relatively constant, although the observed changes could have been possible in both directions. During all steady states, glucose uptake showed a stepwise increase and the glucose concentration showed a corresponding decrease. Glucose uptake potential and glucose concentration in the chemostat were inversely proportional. The ATP content of the cells varied up to 33% during the steady state, but did not show a general trend. After long cultivation in all chemostats, plate counts on both agars dropped to values less than 20% of the original steady-state level. These decreases were due to an inability of the cells to grow on agar plates, not to a lack of vitality of the cells in the chemostat. This study showed that even during shorter chemostat runs, e.g., 1 week, changes in important parameters with the steady state must be expected, especially in the uptake potential and the concentration of the limiting substrate.

During the last three decades the chemostat has become a valuable and well-established tool in microbial physiology and ecology (5, 25, 41, 43, 44). Despite the extensive use and sound mathematical description of the chemostat (1, 16, 36), several basic biological questions remain to be answered.

The first and most basic question is that of the stability and duration of the so-called steady state, i.e., the final state of operation when the biomass and the chemical environment in the chemostat are considered to be constant. This constancy is the basic assumption for all steady-state kinetics (16). However, there are only a few general and somewhat contradictory comments on the constancy of the biomass and the concentration of the limiting substrate during long-term steady states in the literature (16, 31, 41).

The second question concerns the biological activity of the cultured microorganisms and their interaction with the chemical environment. Although this aspect is essential to the self-adjusting behavior of the continuous culture, it has often been neglected. However, changes in the activity of microorganisms during a prolonged steady state are well known (8, 9, 23, 28, 29, 31).

The third question concerns the viability of the cells in the chemostat and the reduction of their viability at low growth rates (34, 35, 41, 42). Whether this reduction of viability reflects dead cells in the chemostat or is an artifact of the viability determination is uncertain (5).

The ecological focus of this study was the investigation of the behavior of populations of microorganisms under prolonged carbon and energy limitation. These environmental conditions, often prevailing in pelagic aquatic habitats, provide a severe challenge to microorganisms (20, 27, 43). In this respect the substrate uptake and viability of the stressed organisms are of special interest.

Cytophaga johnsonae was chosen as a test organism to study the above-mentioned questions of general and ecological concern. This gliding bacterium is well known for its occurrence in a variety of aquatic habitats (2, 7, 11) and represents a group of microorganisms known for its phylogenetic distance from the *Enterobacteriaceae* (38). This phylogenetic distance not only is important for the ecology of free-living bacteria, but should also allow a further generalization of the results (32, 43).

Long-term studies of glucose-limited chemostat cultures of *C. johnsonae* were undertaken to investigate both the physiological and the ecological aspects. The biological activity of the

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cells in the chemostat was monitored by measuring their glucose uptake potential, the residual glucose concentration, their ATP content, and their ability to grow on two different agar media.

MATERIAL AND METHODS

Organism. *C. johnsonae* C-21, a freshwater isolate with psychrotrophic growth characteristics, has been described previously (21). A G+C content of 34% and a DNA molecular weight of 5×10^9 , recently reported by Güde (personal communication), supported the classification of this strain.

Media. All liquid culture experiments were done in a defined, phosphate-buffered, mineral medium (21) containing 450 mg of glucose per liter (2.5 mmol/liter) as the sole organic carbon and energy source. The medium was sterilized as a whole by membrane filtration after adjusting the pH to 7.1 with sodium hydroxide. For plate count experiments the following two agar media were used: (i) for G2 agar, the phosphate buffer of the mineral medium (21) was autoclaved with 15 g of agar (Merck; no. 1614) per liter, and filter-sterilized glucose (2 g/liter) and the rest of the salts were added afterward; (ii) for N8 agar, 8 g of nutrient broth (Merck; no. 5443) per liter was autoclaved with 15 g of agar per liter in distilled water.

Culture conditions. The continuous cultures were carried out in conical glass reaction vessels (diameter, 100 mm; length, 370 mm; Sovirel). The working volume of a reaction vessel was 1 liter in a total volume of 2.2 liters, and the aeration rate was 100 liters per h. The chemostats were fed from 20-liter glass reservoirs bottles by peristaltic pumps (Gilson), and the flow rates were checked daily. Details of construction and operation are given elsewhere (M. G. Höfle, Ph.D. thesis, University of Freiburg, Freiburg, Federal Republic of Germany, 1981). The pH of the medium decreased during growth by less than 0.1 unit. Temperature was maintained at 20°C ($\pm 0.1^\circ\text{C}$) by immersing the cultures in a water bath. The chemostats were inoculated with 10 ml of a logarithmically growing preculture grown in the same glucose mineral medium and at the same culture conditions. After logarithmic growth and 8 h of stationary phase in the reaction vessel, the continuous operation of the cultures was started. This starting point is indicated in all graphs by the origin of the generation marking. The absence of contaminants during the continuous culture was checked by microscopic observation and plating on N-8 agar. Contaminants could be positively and rapidly detected on the nutrient agar plates because of the yellow color of the *C. johnsonae* colonies and the flexirubin positivity of the strain, a characteristic of all freshwater *Cytophaga* spp. (19). In general the chemostat cultures of *C. johnsonae* fulfilled all of the operational criteria listed by Veldkamp (44) for a homogeneous continuous culture. In particular, no wall growth and clumping was observed during the steady state. Glucose limitation was tested by doubling the glucose concentration in the reservoir, which resulted in the expected doubling of the biomass in the chemostat.

Analyses. Optical densities (OD) were read in a Zeiss P1 4 spectrophotometer at 578 nm with a 1-cm cuvette. Estimates of dry weight from the OD values were done on the basis of a standard curve that related the OD

values of different cultures to their dry weight. A single, standard curve proved adequate, regardless of the growth rate or culture type (batch or chemostat).

For the ATP analysis, 200 μl of a cell suspension was withdrawn from the culture and injected into 800 μl of boiling Tris-buffered EDTA (20 mmol of Tris per liter–2 mmol of EDTA per liter, adjusted with sulfuric acid to pH 7.75) and boiled for 3 min (30). After cooling to room temperature, these extracts were frozen at -20°C until further analysis. The ATP content of the extracts was determined with a Lumacounter 2080 (Abimed) with highly purified luciferase (Lumit PM grade; Abimed) dissolved in 25 mmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per liter (pH 7.75; Abimed). The internal standardization technique of Strehler (39) was used for calibration and calculation of the absolute amount of ATP in the extracts.

A modified glucose oxidase method (3) was used for the determination of glucose values higher than 5 mg/liter in the culture suspension; for lower values a modification of the ATP differential method (6) with a purified hexokinase (Sigma Chemical Co.; H 5500) and the above-described ATP measuring procedure was used (for details, see Höfle, Ph.D. thesis).

Plate counts. The culture suspension was diluted stepwise (100 times) with sterile mineral medium without glucose to estimate the CFU. Then 100 μl of an appropriate dilution was spread on the relevant agar plate and incubated for 7 to 10 days at 20°C. For optimal plate count estimates the CFUs were calculated from eight replica plates as recommended by Postgate (33). To calculate the specific CFUs, the OD value of the undiluted suspension and the above-mentioned dry weight calibration curve were used.

Uptake assay. For all uptake assays, the cells were washed as follows: a cell suspension was filtered (vacuum, 0.1 bar) onto a Nuclepore filter (47-mm diameter, 0.2- μm pore size; Nuclepore Corp.). The cells on the filter were washed two times with 10 ml of sterile mineral medium and were suspended in mineral medium at a concentration of 1 to 2 mg (dry weight) per ml. This washing procedure (necessary for standardization and removal of unlabeled glucose) was far superior to centrifugation, since washed cells showed no significant difference in uptake compared with unwashed cells. The uptake assay was started by adding 1 ml of the washed cell suspension to 50 μl of [U - ^{14}C]glucose solution (Amersham Corp.; specific activity, 1.5 mCi/mg; final assay concentration, 1 $\mu\text{mol/liter}$ [180 $\mu\text{g/liter}$]). The assay was done in test tubes (13 by 140 mm) in a 20°C water bath; all components were at that temperature from the beginning of the assay. After 7.5, 15, 30, 45, and 60 s, 100 μl of the reaction mixture was placed on a membrane filter (25-mm diameter, 0.65- μm pore size, cellulose nitrate; Sartorius), filtered immediately, and washed with 8 ml of mineral medium. Immediately after filtration the membrane filters were put in scintillation vials filled with a dioxan-based scintillation cocktail. The counts per minute of the filters were determined with a Beckman LS 7500 scintillation counter. The absolute uptake rate was calculated from the slope of the least-squares linear regression line for the five pairs (counts per minute, time). Typical results of the uptake assay showed very good linearity and a correlation coefficient greater than 0.99. To calculate the specific up-

take rate, the OD of the washed cell suspension was used as a measure of the biomass. Specific uptake rates in all experiments were measured under the above-indicated conditions; they therefore represent potential uptake rates of the cells and not actual uptake rates in the culture.

RESULTS

Long-term changes in the biomass concentration. The constancy of the biomass X is (according to the steady state condition $dX/dt = 0$) the basic criterion for reaching and stabilizing the steady state in a continuous culture (16). OD measurements for *C. johnsonae* showed a constant and growth rate-independent relationship with dry weight (Höfle, Ph.D. thesis). The optical density of different continuous cultures could therefore be used to estimate the long-term constancy of the biomass. Representative results for a high (75% $\mu_{\max}$) and a low (12.5% $\mu_{\max}$) dilution rate (D) are summarized in Table 1. These data show that biomass is generally stable within a certain range ($\pm 5\%$) during a several hundred-hour period, but at certain times significant changes occur (see Fig. 1 and 5). These changes are relatively rare and stochastic events. They only occurred once in all chemostat runs and showed both increase and decrease of the cellular yield. The third possibility was also realized: the biomass showed no significant changes over more than 2,000 h (Table 1, run CH/II-6). The biomass change was at most 24.2% of the initial steady-state value, which is relatively small in comparison to the other measured parameters.

Long-term changes in glucose uptake and glucose concentration. The glucose uptake and the glucose concentration in the chemostat were first followed in a chemostat with a high dilution rate ($D = 0.15 \text{ h}^{-1}$, 75% $\mu_{\max}$). The results show a stepwise increase of the glucose uptake potential of the cells and a corresponding decrease of the glucose concentration in the reaction vessel (Fig. 1). During this run (CH/I-17), five plateau or steady-state values for glucose uptake and concentration were observed. The mean values

FIG. 1. Long-term changes of OD (+), glucose concentration (x), and glucose uptake potential (■) during the steady state of a fast-growing glucose-limited chemostat culture (D , 0.15 h^{-1} ; run CH/I-17) of *C. johnsonae*.

of these average steady-state levels are given in Table 2. The first plateau for the glucose concentration is not shown in Fig. 1 because of scale problems, and the first uptake plateau is only represented by its final point. Details of the transient and the first steady state are described elsewhere (22). The relationship between the glucose uptake potential of the cells and the glucose concentration in the chemostat seems to be inversely proportional and could be described by the following equation (formula 1): $\hat{s} = a/v_{\text{gluc}}$, where $\hat{s}$ is the glucose concentration in the chemostat, a is the proportionality constant, and v_{gluc} is the glucose uptake potential. Figure 2 shows that the hyperbolic dependence of the glucose concentration on the glucose uptake potential and that the data presented in Table 2 fit well into formula 1 when the mean proportionality constant a is used. A second test for the validity of formula 1 is the linear transformation of the data according to the following equation (formula 2) (Fig. 2, insert): $\hat{s} = a(1/v_{\text{glu}}) + b$, where b is the y intercept. It can be seen by this

TABLE 1. Long-term stability of the biomass concentration during continuous cultures of *C. johnsonae* at a high (D , 0.15 h^{-1}) and a low (D , 0.03 h^{-1}) dilution rate

Run	D (h^{-1})	Time (h)	OD at 580 nm $\pm$ %SD	Biomass (mg [dry wt] per liter)	% Difference
CH/I-17	0.150	38–648	0.270 ± 4.6	177.6	
CH/I-17	0.150	720–1,244	0.336 ± 4.0	220.6	24.2
CH/I-22	0.030	278–619	0.187 ± 3.2	123.0	
CH/I-22	0.030	686–1,295	0.216 ± 5.2	141.6	15.1
CH/II-6	0.030	287–1,486	0.192 ± 6.5	126.3	
CH/II-6	0.030	1,606–2,068	0.199 ± 4.8	130.8	3.6
CH/II-7	0.030	320–704	0.207 ± 5.7	136.0	
CH/II-7	0.030	724–1,156	0.177 ± 4.1	116.3	–15.5

TABLE 2. Glucose uptake potential and glucose concentration during different steady states in a continuous culture of *C. johnsonae* (D , 0.15 h^{-1})

Steady state (h)	Glucose uptake (nmol/min per mg $\pm$ %SD)	Residual glucose concn (mg/liter $\pm$ %SD)	Proportionality constant a^a
40–190	0.101 ± 17.6	22.7 ± 11.3	2.29
190–480	0.383 ± 5.1	7.43 ± 4.1	2.84
550–670	0.778 ± 4.0	2.61 ± 5.6	2.03
770–1,000	1.526 ± 4.1	1.24 ± 13.2	1.90
1,000–1,360	2.464 ± 5.1	0.64 ± 12.8	1.57

^a The mean of a is $2.13 \pm 22.5\%$.

transformation that all plateau values lie quite close to the regression line. This linear relationship between the glucose residual concentration δ in the chemostat and the inverse of the uptake potential was highly significant and indicates that the slope a , which gives the proportionality constant from formula 1, is constant within its statistical limits. Whether there is an additional term b could not be determined since the confidence limits of the regression line include the origin. It must be pointed out here that all uptake potentials were measured at a constant and subsaturating substrate concentration (see above and reference 22).

The stepwise decrease in the substrate concentration δ during this chemostat run also correlates with a stepwise decrease in the saturation constant for growth k_s of the cells. According to Herbert et al. (16), both parameters depend only upon the dilution rate D and the maximum growth rate $\mu_{\max}$ as other variables. Since these variables are constant during a chemostat run, k_s is directly proportional to δ , and when D is 0.15 h^{-1} and $\mu_{\max}$ is 0.2 h^{-1} the saturation constant k_s is one-third of the substrate concentration δ .

The occurrence of similar stepwise changes in the glucose uptake potential at a low growth rate (D , 0.03 h^{-1}) when a different, high-affinity glucose uptake system is working (22) can be seen in Fig. 3. Results from two independent chemostat runs show that the uptake potentials are nearly the same within the first 500 h. After the first steady state was reached, the uptake in run I increased more slowly than that in run II, where a maximum uptake of 7.3 nmol of glucose per min per mg (dry weight) was reached after 1,000 h. This rate was twice as high as the final rate reached in run I after 1,800 h. These differences indicate that the glucose uptake potential of a *C. johnsonae* population is only reproducible in the first part of a chemostat run, when the first steady state is reached after the transient state (22). Random events occur later which lead to unpredictable increases in the uptake potential.

FIG. 2. Dependence of glucose concentration and uptake potential during the five different steady state levels in the fast-growing chemostat (D , 0.15 h^{-1}). The dotted line was calculated according to formula 1 ($a = 2.13$). The insert shows linearized plot (correlation coefficient $r = 0.997$) according to equation 2.

Long-term effects on the ATP content. Despite high variance, the ATP content of the cells in the fast-growing chemostat (D , 0.15 h^{-1}) showed no significant changes during the first 800 h (Fig. 4). The ATP content remained at an average of 1.25 ng of ATP per μg (dry weight) $\pm 8.2\%$ and was not significantly different from the ATP content of cells from the logarithmic phase of a batch culture (1.235 ng of ATP per μg [dry weight] $\pm 3.6\%$). A significant decreasing trend in the ATP content could be observed after 800 h, ending

FIG. 3. Long-term changes of OD (+) and glucose uptake potential during the steady states of two different chemostat runs (run I = run CH/II-6 [■] and run II [□]) at a low dilution rate (D , 0.03 h^{-1}) with *C. johnsonae*.

FIG. 4. Long-term changes of OD (+), ATP per milliliter (■), and ATP per milligram (dry weight) (x) during the steady state of a fast growing glucose-limited chemostat culture (D , 0.15 h^{-1}) of *C. johnsonae*.

finally after 1,300 h with an ATP content of 0.89 ng of ATP per μg (dry weight).

The pattern of the ATP content in the slowly growing chemostat (D , 0.03 h^{-1}) was more complex (Fig. 5). In this culture, the transient state was much longer (300 h [22]), and the ATP values before 300 h were thus not steady state values. After a constant phase between 300 and 600 h with an average ATP content of 1.10 ng of ATP per μg (dry weight) $\pm 2.4\%$, the ATP content showed some oscillation. This oscillation, characterized by two maxima at 1.46 ng of ATP per μg (dry weight) and a minimum at 0.90 ng of ATP per μg (dry weight), showed that the ATP content in long-term chemostat runs can

FIG. 5. Long-term changes of OD (+), ATP per milliliter (■), and ATP per milligram (dry weight) (x) during the steady state of a slow-growing glucose-limited chemostat culture (D , 0.03 h^{-1} , run CH/1-22) of *C. johnsonae*.

vary on a random pattern by one-third of the original steady-state value.

Long-term effects on the CFU. The specific CFU was chosen as a parameter to show the changes of the absolute CFUs in the chemostats as a function of time and of the agar used. This parameter indicates the number of CFUs produced per milligram (dry weight) and allows comparison of CFUs independent of the optical density in the chemostat. The only assumption made is that the cells do not change shape during the steady state, which was verified for this organism by microscopic observation. The specific CFUs on both agar media were stable for a long time in the fast-growing chemostat (D , 0.15 h^{-1}) (Fig. 6). The average value in this phase was only slightly higher (20%) than the average value for cells of the logarithmic phase of a batch culture. It could be shown for the latter that there was no significant difference between the CFUs and the direct counts (measured on Nuclepore filters by the acridine orange method of Hobbie et al. [18]) of the cell suspension, i.e., all cells in this phase were viable. After 600 and 750 h the specific CFUs on G2 and N8 agar decreased rapidly to 11 and 14%, respectively, of the first steady-state level.

During the first 300 h in the slowly growing chemostat (D , 0.03 h^{-1} , Fig. 7), before the steady state was reached, the specific CFUs increased strongly due to a fragmentation of the cells in this phase (Höfle, Ph.D. thesis). After a sharp decrease in the second half of that transient state, the specific N8 agar CFUs leveled off for a short time before they decreased to a value only 19% of the first steady-state level. In Fig. 7 the specific G2 agar CFUs departed from their

FIG. 6. Long-term changes of OD (+) and specific CFUs per milligram (dry weight) on N8 (x) and G2 (□) agar during the steady state of a fast-growing glucose-limited chemostat culture (D , 0.15 h^{-1}) of *C. johnsonae*.

FIG. 7. Long-term changes of OD (+) and specific CFUs per milligram (dry weight) on N8 (x) and G2 (□) agar during the steady state of a slow-growing glucose-limited chemostat culture (D , 0.03 h^{-1}) of *C. johnsonae*.

usual behavior of following the N8 agar CFUs on a slightly lower level; they showed a sudden marked increase between 600 and 750 h. However, after 750 h the CFUs on G2 agar also decreased to a level as low as that of the N8 agar CFUs. In general the CFUs showed a downward trend in all chemostat cultures with prolonged culture time. This trend was more pronounced in slowly growing than in fast-growing chemostats (Fig. 6 and 7).

DISCUSSION

Conservative parameters. The parameters investigated can be divided according to their responses into two groups: (i) conservative parameters showing slight changes in random directions and (ii) nonconservative parameters showing large changes (>30%) in the original steady-state values in only one direction. The most apparent conservative parameter during the steady state in the continuous culture was biomass (Table 1). In all recorded chemostats, the biomass concentration during the steady state changed only once, if at all, and the order of magnitude of that change was less than one-fourth of the original value. That these changes occurred at a random time and in a random direction is a strong indication that the cell yield is under no specific selection pressure in these glucose-limited chemostats.

The gross biochemical composition of the cells (expressed as total protein, carbohydrate, lipid, and RNA) did not change significantly during the steady state (Höfle, Ph.D. thesis). The influence of the dilution rate on the gross biochemical composition of *C. johnsonae* was small, except that RNA content decreased with decreasing dilution rate, as has been well docu-

mented for some bacteria (4, 13, 15). The elementary composition (C, H, N) of the cells was not influenced by the duration of the steady state or by the dilution rate (Höfle, Ph.D. thesis).

The ATP content of *C. johnsonae* varied more with time at the steady state than with changes in the dilution rate (Fig. 4 and 5). The ATP content could change up to 33% of the initial value during steady-state growth. Different growth rates, ranging from $6\% \mu_{\max}$ to $\mu_{\max}$, resulted only in changes up to 25% of the initial content of ATP (Höfle, Ph.D. thesis). However, these relatively small changes and the range of the ATP contents indicate that the cellular ATP content should be regarded as a conservative parameter, despite the relatively large scatter in the data which has also been observed by other investigators (10, 14). Cellular ATP content is a rather complex and sensitive value (26), probably reflecting short-term variations in continuous culture due to unknown metabolic or operationally induced oscillations (14) and is therefore not very apt as a biomass parameter in chemostats.

Nonconservative parameters. In contrast to biomass, bioactivity is a nonconservative parameter. This can be seen especially in the uptake of glucose, the limiting substrate in these continuous cultures (Fig. 1). The stepwise increase of the glucose uptake potential during prolonged continuous culture, showing an overall 25-fold increase, can be interpreted as an improvement of the glucose uptake mechanisms or their regulation by mutants. A similar phenomenon has been studied for the amino acid uptake of *Escherichia coli* in amino acid-limited chemostats. It has been shown that an increased uptake potential was caused by an increase in the number of protons taken up during amino acid uptake via a proton symport mechanism (8).

Because of the inverse proportionality between substrate uptake potential and substrate concentration, an increase in the uptake potential causes a decrease in the substrate concentration (Fig. 1). For a certain period of time, between different plateaus, a disturbance of the steady state occurs in substrate concentration and uptake without any significant effect on the biomass. Thus, a steady state in respect to the substrate flux is only achieved at the five plateau situations (Table 2) and not during the transitions between them. This inverse relationship between substrate uptake potential of the bacteria and the ambient substrate concentration also shows how strongly coupled the levels of the limiting substrate are to the activity of heterotrophic microorganisms.

The steep decreases of the specific CFUs during both long-term runs at different dilution rates (Fig. 6 and 7) cannot be explained by a

simple decrease in the vitality of the cells. The high ATP content of the cells and the high glucose uptake potential of the cells indicate that a large part of the population did not die when the specific CFU values dropped more than 80%. Also, if one assumes that almost 90% of the cells in the chemostat are dead, the viable part of the population would have to grow faster than 1 doubling per h in the chemostat with the high dilution rate ($D = 0.15 \text{ h}^{-1}$), according to the correction equation of Tempest (40). Such a high growth rate has never been reported for a bacterium growing at 20°C in a glucose mineral medium without any additional precursor compounds. It can therefore be assumed that during long-term runs the bulk of the *C. johnsonae* population loses its ability to grow on agar plates without losing its vitality or metabolic activity in the continuous culture.

The inability of large parts of the *C. johnsonae* population to grow on agar plates after a certain time in a steady state of a glucose-limited continuous culture is also interesting from an ecological point of view, since it is well known that only a very small fraction of the total number of bacteria in a water sample is able to grow on agar plates (24). The most common explanation for this behavior of free-living aquatic bacteria is that the agar is too selective for the growth of most bacteria. This is certainly not the reason for the drop of the CFUs in the chemostat cultures because the medium remained the same during the whole experiment. Only an intrinsic change in the population could be responsible for this behavior.

A hint for a possible mechanism could be that the specific CFU on N8 agar drops as the glucose uptake rate showed a great absolute increase in the fast growing chemostat (3rd to 4th plateau in Fig. 1 and 6). This would lead to the explanation that, after a certain high uptake potential is reached, the cells become sensitive to the high substrate concentration on the agar plate and most cells are unable to multiply due to an oversupply of nutrients. This would be similar to the substrate-accelerated death reported by Postgate and Hunter (35). However, it can be seen in the curve of the specific CFUs on G2 agar in the slowly growing culture (Fig. 7) that the responses of *C. johnsonae* could also be the opposite. The strong increase of the CFUs on G2 agar between 550 and 750 h when the glucose uptake potential is already high or rising (Fig. 3) and when glucose should be a traumatic substrate cannot be explained as substrate-accelerated death caused by an increased uptake potential. It is therefore obvious that the uptake potential for the limiting substrate is not the only factor influencing the ability of the cells to grow on agar plates. Despite these puzzling findings,

the results show that long-term chemostat runs can be used for experimental studies of the reasons that most aquatic bacteria do not grow on agar plates.

General conclusions. Changes in important parameters can occur during steady-state growth in chemostats. At higher growth rates these changes can occur within 1 week after reaching steady state and not within 1 month as stated by Herbert et al. (16) and Tempest (41). These long-term changes occur in addition to short-term physiological regulations which are especially important for chemostat studies in which more than one transport system is present for the limiting substrate (22).

Changes in the residual concentration of the limiting substrate, a basic parameter of the chemical environment, must be expected due to changes in substrate uptake potential while the biomass remains constant. The chemical environment of the chemostat is therefore much more time dependent than the biomass. The assessment of the residual concentration of the limiting substrate in the chemostat is therefore a more sensitive parameter for measuring changes during a steady state than the biomass. Monitoring the substrate concentration, usually omitted because of analytical difficulties (37), would allow the verification of the constancy of the substrate concentration. As shown in this study, this concentration, usually assumed to be low and constant, can change greatly.

Harder et al. (12) suggested that selection pressure is strongest on the rate-limiting step of the substrate-limited growth in a chemostat. If this is true, the stepwise increase of the uptake potential of the limiting substrate indicates that the uptake of the limiting substrate is the rate-limiting step during growth of *C. johnsonae* in chemostat culture. This is in accordance with the findings of Herbert and Kornberg (17), who showed the interdependence of substrate transport and growth rate in chemostat cultures with *E. coli*. Koch and Wang (28) also reported recently that during a long-term glucose-limited chemostat run with *E. coli*, the saturation constant for growth k_s increased, whereas the maximum growth rate decreased. All of these results indicate that there is strong regulation and selection in the chemostat for the saturation constant for growth, k_s , which in turn is closely related to the saturation constant k_m of the uptake systems for the limiting substrate (22). A quantitative relationship between these two saturation constants remains to be developed and should be one of the goals of future chemostat research.

These results show that the time independence of microorganism populations in chemostats should be carefully monitored and that, for longer chemostat runs, the history of the popula-

tion becomes more and more important the longer the chemostat run lasts. This study also shows that time course chemostat experiments deserve more attention because they can help to elucidate the evolution and ecology of microorganisms and improve our understanding of general chemostat data.

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LITERATURE CITED

- Bazin, M. J. 1981. The theory of continuous culture, p. 27-62. In P. H. Calcott (ed.), *Continuous cultures of cells*, vol. 1. CRC Press, Boca Raton, Fla.
- Bell, C. R., M. A. Holder-Franklin, and M. Franklin. 1982. Correlation between predominant heterotrophic bacteria and physicochemical water quality parameters in two Canadian rivers. *Appl. Environ. Microbiol.* 43:269-283.
- Bergmeyer, H. U. 1974. *Methoden der enzymatischen Analyse*. 3. Aufl. Verlag Chemie, Weinheim.
- Bovallius, A. 1979. Morphological and chemical characteristics of a *Cytophaga* sp. grown under conditions of magnesium excess and magnesium limitation. *J. Gen. Microbiol.* 113:137-145.
- Calcott, P. H. 1981. Continuous culture: where it came from and where it is now, p. 1-12. In P. H. Calcott (ed.), *Continuous cultures of cells*, vol. 1. CRC Press, Boca Raton, Fla.
- Cavari, B. Z., and G. Phelps. 1977. Sensitive enzymatic assay for glucose determination in natural waters. *Appl. Environ. Microbiol.* 33:1237-1243.
- Christensen, P. J. 1977. The history, biology and taxonomy of the *Cytophaga* group. *Can. J. Microbiol.* 23:1599-1653.
- Collins, S. H., A. W. Jarvis, R. J. Lindsay, and W. A. Hamilton. 1976. Proton movements coupled to lactate and alanine transport in *Escherichia coli*: isolation of mutants with altered stoichiometry in alanine transport. *J. Bacteriol.* 126:1232-1244.
- Cox, C. E., and T. C. Gibson. 1974. Selection for high mutation rates in chemostats. *Genetics* 77:169-184.
- Forrest, W. W. 1967. Energetics of activation and uncoupled growth in *Streptococcus faecalis* and *Zymomonas mobilis*. *J. Bacteriol.* 94:1459-1463.
- Güde, H. 1980. Occurrence of *Cytophagas* in sewage plants. *Appl. Environ. Microbiol.* 39:756-763.
- Harder, W., J. G. Kuenen, and A. Matin. 1977. Microbial selection in continuous culture, a review. *J. Appl. Bacteriol.* 43:1-24.
- Harder, W., and H. Veldkamp. 1967. A continuous culture study of an obligately psychrophilic *Pseudomonas* species. *Arch. Microbiol.* 59:123-130.
- Harrison, D. E. F., and H. H. Topiwala. 1974. Transient and oscillatory states of continuous culture. *Adv. Biochem. Eng.* 3:167-219.
- Herbert, D. 1958. Continuous culture of microorganisms, some theoretical aspects, p. 45-52. In *Continuous cultivation of microorganisms*, a symposium. Publishing House of the Czechoslovak Academy of Sciences, Prague.
- Herbert, D., R. Elsworth, and R. C. Telling. 1956. The continuous culture of bacteria; a theoretical and experimental study. *J. Gen. Microbiol.* 14:601-622.
- Herbert, D., and H. L. Kornberg. 1976. Glucose transport as rate-limiting step in the growth of *Escherichia coli* on glucose. *Biochem. J.* 156:477-480.
- Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33:1225-1228.
- Hirsch, I., and H. Reichenbach. 1981. The Cytophaga-like bacteria: a search for key characters, p. 145-151. In H. Reichenbach and O. B. Weeks (ed.), *The flavobacterium-cytophaga group*. GBF Monograph series no. 5. Verlag Chemie International, Weinheim.
- Hirsch, P. 1979. Life under conditions of low nutrient concentrations (group report), p. 357-372. In M. Shilo (ed.), *Strategies of microbial life in extreme environments*. Verlag Chemie, Weinheim.
- Höfle, M. G. 1979. Effects of sudden temperature shifts on pure cultures of four strains of freshwater bacteria. *Microb. Ecol.* 5:17-26.
- Höfle, M. G. 1982. Glucose uptake of *Cytophaga johnsonae* studied in batch and continuous culture. *Arch. Microbiol.* 133:289-294.
- Horiuchi, T., J. Tomzawa, and A. Novick. 1962. Isolation and properties of bacteria capable of high rates of β -galactosidase synthesis. *Biochim. Biophys. Acta* 55:152-163.
- Jannasch, H. W., and G. E. Jones. 1959. Bacterial populations in sea water as determined by different methods of enumeration. *Limnol. Oceanogr.* 19:716-720.
- Jannasch, H. W., and R. I. Mateles. 1974. Experimental bacterial ecology studies in continuous culture. *Adv. Microb. Physiol.* 11:165-212.
- Karl, D. M. 1980. Cellular nucleotide measurements and applications in microbial ecology. *Microbiol. Rev.* 44:739-796.
- Koch, A. L. 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. *Adv. Microb. Physiol.* 6:147-217.
- Koch, A. L., and C. H. Wang. 1982. How close to the theoretical diffusion limit do bacterial uptake systems function? *Arch. Microbiol.* 131:36-42.
- Kubitschek, H. E. 1970. Introduction to research with continuous cultures. Prentice-Hall, Inc., Englewood Cliffs, N.J.
- Lundin, A., and A. Thore. 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. *Appl. Microbiol.* 30:713-721.
- Novick, A., and L. Szillard. 1950. Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 36:708-719.
- Osche, G. 1975. Die vergleichende Biologie und die Beherrschung der Mannigfaltigkeit. *Biol. Unserer Zeit* 5:139-146.
- Postgate, J. R. 1969. Viable counts and viability. *Methods Microbiol.* 1:611-628.
- Postgate, J. R. 1973. The viability of very slow-growing populations: a model for the natural ecosystem. *Bull. Ecol. Res. Comm. Stockholm* 17:287-292.
- Postgate, J. R., and J. R. Hunter. 1964. Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. *J. Gen. Microbiol.* 34:459-473.
- Powell, E. O. 1967. The growth rate of microorganisms as a function of substrate concentration, p. 34-55. In E. O. Powell, C. G. T. Evans, R. E. Strange, and D. W. Tempest (ed.), *Microbial physiology and continuous culture*, 3rd International Symposium. Her Majesty's Stationery Office, London.
- Rhee, G. Y. 1980. Continuous culture in phytoplankton ecology. *Adv. Aquat. Microbiol.* 2:151-203.
- Stanier, R. Y., E. A. Adelberg, and J. L. Ingraham. 1976. *The microbial world*. Prentice-Hall, Inc., Englewood Cliffs, N.J.
- Strehler, B. L. 1968. Bioluminescence assay: principles and practice. *Methods Biochem. Anal.* 16:341-356.

40. **Tempest, D. W.** 1969. The continuous cultivation of microorganisms. I. Theory of the chemostat. *Methods Microbiol.* 2:249-276.
41. **Tempest, D. W.** 1970. The place of continuous culture in microbiological research. *Adv. Microb. Physiol.* 4:223-241.
42. **Tempest, D. W., D. Herbert, and P. J. Phipps.** 1967. Studies on the growth of *Aerobacter aerogenes* at low dilution rates in a chemostat, p. 240-254. *In* E. O. Powell, C. G. T. Evans, R. E. Strange, and D. W. Tempest (ed.), *Microbial physiology and continuous culture*. Her Majesty's Stationery Office, London.
43. **Tempest, D. W., and O. M. Neijssel.** 1978. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. *Adv. Microb. Ecol.* 2:105-153.
44. **Veldkamp, H.** 1976. Continuous culture in microbial physiology and ecology, p. 1-68. Meadowfield Press, Ltd., Durham, England.