Journal of Industrial Microbiology (1996) 17, 235–252

Physiologic, morphologic and behavioral responses of perpetual cultures of *Caulobacter crescentus* to carbon, nitrogen and phosphorus limitations

ER Felzenberg, GA Yang, JG Hagenzieker[1] and JS Poindexter

Department of Biological Sciences, Barnard College, Columbia University, New York, NY 10027–6598, USA

Perpetual cultures of *Caulobacter crescentus* were maintained in a chemostat and provided with media that provided glucose, glutamate, ammonium chloride, and Na/K phosphate as sources, respectively, of C, of C and N, of N and of P. All cultures were maintained at the same relative flow rate, f/V, so that reproductive rate was constant. As the C : N : P ratio was shifted from a balanced ratio of 100 μg C: 9–10 μg N: 1 μg P, the cells modulated their rates of uptake, reducing the rates of uptake of excess element-sources and accelerating the uptake of potential sources of the limiting element. The change in N-limited cells was the greatest, resulting in a nearly 150-fold increase in rate of uptake of amino acids. Poly-β-hydroxybutyrate or polyphosphate was stored whenever C or P, respectively, was not the limiting element. Direct measurement of cell and stalk surface areas on purified peptidoglycan sacculi of P-limited cells revealed that the surface of both swarmer and stalked cells, as well as the stalk surface, participated in the accelerated rate of phosphate uptake. Swarmers from N-limited populations were the only cells that exhibited chemotactic responsiveness—to methionine, NH_4Cl and glutamate, but not to glucose or phosphate—sufficient to be detectable in a microcapillary assay. In populations that were C-limited and provided with ammonium ions as the principal source of N, morphogenesis and reproduction deteriorated and a steady state could not be maintained. Generally, the responses of *C. crescentus* were appropriate to manage unbalanced nutrient supplies, but this oligoheterotrophic organism did not tolerate excesses of inorganic nutrients when limited for its sources of C, of energy, and of ammonium acceptor.

Keywords: C : N : P ratio; *Caulobacter crescentus*; cell surface area; chemostat; chemotaxis; uptake

Introduction

Prolonged cultivation of microbial populations occurs in various commercial and municipal applications in bioreactors, fermentors, and sewage and water treatment facilities. In such settings, as in most natural habitats, microorganisms are usually challenged to survive and multiply at the expense of mixtures of nutrients and in the presence of a variety of microorganisms. To some degree, these features can be provided in laboratory-maintained perpetual cultures. Such cultures, principally chemostats, of monotypic and of mixed microbial populations, with single and multiple limitations, are useful in evaluating various aspects of potentially applicable microbe-catalyzed processes, such as suitability of specific organisms for specific processes, tolerance of the microbial population for toxic substances that can be cometabolized with supportive nutrients, influences of physicochemical factors on biomass or product yields, etc. The relevance of fundamental perpetual culture studies in microbial physiology, ecology and population dynamics to industrial applications was a central topic of a recent symposium on microbial growth kinetics, whose discussions were published together [see 23 and accompanying articles].

Studies with perpetual cultures that provide more than one source of carbon and energy or of nitrogen have usually revealed that bacteria and yeasts are capable of utilizing mixtures of nutrients by employing mixtures of enzymes that, in batch-cultivated populations, do not act together. In chemostat cultures, this can occur even though: (a) some of the enzymes are repressed during batch cultivation by the presence of other enzymes' substrates [8,11,15]; (b) some of the enzymes are repressed or inactivated in the presence of high concentrations of their own substrates [12,32] or derepressed when their own product concentration is low [60]; or (c) some of the metabolizable substances are generally toxic in batch cultures [26]. Laboratory perpetual cultures have also revealed the value of specific microbial properties to microbial competitiveness [21,28,31] and the relative effectiveness of different metabolic pathways for degradation of toxic and potentially hazardous substances [10].

Under perpetually nutrient-poor conditions, microorganisms exhibit diverse physiologic responses that include increased rates and affinities of nutrient uptake systems, derepression of catabolic enzymes and of hydrolytic scavenging enzymes, storage of 'excess' nutrients, and increased capacity for respiration of substrates [reviewed in 16,19,20,57]. Nutrient limitation can also enhance chemotactic responsiveness [58]. Each of these changes would serve to enhance the microorganisms' ability to find, take

Correspondence: JS Poindexter, Department of Biological Sciences, Barnard College, Columbia University, 3009 Broadway, New York, NY 10027-6598, USA
[1]Present address: Department of Pathology, Seattle Children's Hospital, Seattle, WA 98105, USA
Received 7 March 1996; accepted 30 August 1996

up, and utilize diverse nutrients as they became available in the environment.

In natural microbial habitats, microorganisms surely (if for no other reason, as the consequence of their own activities [30]) exist most of the time under conditions of nutrient limitation. However, there seem to be two ends of the limitation-response spectrum. Copiotrophic bacteria are accustomed to periodic floods (feasts) of useable nutrients; they suffer shock and may die if nutrient deprivation (fasting) is prolonged [24]. In contrast, oligotrophic bacteria survive quite well as stable populations in habitats where nutrient availability varies between limiting (fasting) and nothing at all (famine) [39]. Even when famine is relieved, the mixture of nutrients transiently available in the habitat of oligotrophic types may not be balanced relative to the composition and the metabolic needs of the organism.

The purpose of the present studies was to examine the hypothesis that the oligoheterotrophic bacterium *Caulobacter crescentus* would exhibit a repertoire of responses that should enable it to manage successive limitation by each of the major nutrient elements (carbon, nitrogen and phosphorus), and that as the relative abundance of the element sources changed, the cells' appetite would shift toward the limiting nutrient while transiently excessive nutrients were stored intracellularly. In addition to the intracellular storage of excess carbon as poly-β-hydroxybutyrate (PHB) and excess phosphorus as polyphosphate, the repertoire included: for each of the three element sources, an increase in substrate uptake rate; for phosphorus in particular, a morphogenetic response that allowed the cells to enlarge and accommodate large reserves of PHB without decreasing their surface : volume ratio; and, for nitrogen, the development of chemotactic responsiveness to gradients of potential nitrogen sources that demonstrably affected the organism's distribution in a liquid environment. Also, another small insight into the nature of oligotrophy was gained through the finding that, like *Hyphomicrobium* [17], nutrient-limited populations of *C. crescentus* encountered difficulty in managing a relatively high flux of ammonium as the principal source of nitrogen.

Materials and methods

Bacteria

Caulobacter crescentus strain 2NY66R, a non-adhesive revertant of the stalk abscission mutant 2NY66 [37] derived from strain CB2 (ATCC 15252 [36]) was used throughout. This strain has been particularly useful in perpetual cultures because, in contrast to the wild-type parent, it neither clings to the culture vessel wall and inserts, nor does it form rosettes, so that colony-forming units (CFU) are single cells.

Media

Peptone/yeast extract medium, employed for preparation of inocula and for plating for viable counts, contained 0.2% (wt/vol) Bacto peptone, 0.1% (wt/vol) Difco yeast extract, and Bacto agar for plates (1%, wt/vol) and overlayers (0.7%, wt/vol).

Hutner's-imidazole buffered-glucose-glutamate ('HiGg') media prepared with glass-distilled water were employed for all chemostat cultures. Hi basal medium contained 5 mM imidazole as pH buffer, and Hutner's mineral base [7] prepared without vitamins and with additional $CaCl_2$ to provide 1 mM calcium. The pH was adjusted to 7.0 before the medium was autoclaved. Glucose, Na_1-glutamate-H_2O, NH_4Cl, and Na/K-phosphate were filter-sterilized and added to autoclaved Hi basal medium at the concentrations shown in Table 1. As indicated in Table 1, each independent perpetual culture was designated by a letter (J, K, N, C); each successive change in reservoir medium delivered to a culture was regarded as a new 'mode' of the culture and was designated by a Roman numeral (I, II, etc).

Previous studies with this strain and its parent in both chemostat and batch cultures had shown that C-limited cells would store P as inorganic polyphosphate, and P-limited cells would store C as PHB [36,38,42,45]. In batch cultures preliminary to the design of the HiGg media employed in the present studies, limitation was assessed by quantitative analysis of PHB (indicating C excess) and Poly-P (indicating P excess), and by the effect on yield in stationary phase of small increments of glucose, of NH_4Cl, or of Na/K-phosphate. Chemostat and batch cultures were in agreement that, in HiGg media, a ratio of 100 μg C per 9–10 μg N per 1 μg P constituted a nutrient supply in which C, N and P were balanced with each other, and all other nutrients (sulfur, major cations, trace elements) were in excess as long as total carbon was less than 3 mg ml^{-1}. The C : N ratio is similar to that observed for other bacteria, most relevantly for *Hyphomicrobium* X [9].

Perpetual cultivation

Perpetual cultures were maintained in a BioFlo benchtop model C30 chemostat operated at 30°C, stirred at 300 rpm, and aerated with sterile air delivered at 0.8–1.0 L min^{-1}. The flow rate of medium from the 12-L reservoir to the culture vessel was 28.3 ml h^{-1}, and the culture volume was 340 ml for all cultures. This rate of delivery replaced culture volume every 12 h, requiring that cells divided on the average every 8.3 h in order to maintain a constant population in the vessel.

Each perpetual culture (J, K, N, or C; Table 1) was begun with an inoculum of 10 ml of a batch culture grown to late exponential phase in peptone/yeast extract broth, then allowed to grow exponentially in the chemostat vessel for five generations before liquid flow through the vessel was begun. A culture was presumed to have attained a steady state when the turbidity and viable count had remained constant for at least 60 h (five volume replacements); the steady state was characterized by sampling over 7 to 55 generations (typically at least 20 generations in the experiments involving chemotaxis).

Changes in relative nutrient fluxes from one mode (I, II, etc; see Table 1) to the next of a perpetual culture were effected by changing the composition of the medium in the reservoir. Tubing volume between reservoir and vessel was approximately 15 ml; consequently, the newly constituted medium began to reach the culture approximately 30 min after the change was effected in the reservoir.

Table 1 Composition of HiGg media for perpetual cultures

Culture/mode[a]	%, wt/vol:			mM	μg ml^{-1}					Ratio[b]
	glc	glt	NH_4Cl	Pi	glc-C	glt-C	glt-N	NH_3-N	Pi-P	C : N : 1P
J/I	0.05	0.05	0.05	0.15	200	160	37	130	4.7	77 : 36
J/II	0.075	0.075	0.05	0.15	300	241	56	130	4.7	115 : 40
J/III	0.10	0.10	0.05	0.15	400	321	75	130	4.7	153 : 44
J/IV	0.15	0.15	0.05	0.15	600	481	112	130	4.7	230 : 51
J/V	0.05	0.05	0.05	0.05	200	160	37	130	1.6	225 : 104
K/I	0.05	0.05	0.05	0.05	200	160	37	130	1.6	225 : 104
K/II	0.075	0.075	0.05	0.05	300	241	56	130	1.6	338 : 116
N/I	0.20	0.033	0.025	0.29	800	106	25	65	9	101 : 9
N/II	0.20	0.033	0.013	0.29	800	106	25	34	9	101 : 6.6
N/III	0.20	0.033	0.042	0.44	800	106	25	109	13.7	66 : 10
N/IV	0.10	0.10	0.025	0.48	400	318	75	65	14.9	48 : 9
N/V	0.10	0.10	0.025	0.12	400	318	75	65	3.7	194 : 38
N/VI	0.10	0.10	0.025	0.48	400	318	75	65	14.9	48 : 9
C/I	0.22	0.0374	0.0107	0.323	880	120	28	28	10	100 : 5.6
C/II	0.275	0.0374	0.0107	0.404	1100	120	28	28	12.5	106 : 4.5
C/III	0.22	0.0748	0.0213	0.180	880	240	56	56	5.6	200 : 20

[a]Each perpetual culture initiated by inoculation of sterile medium in the vessel was designated by a letter. Each time the composition of the reservoir medium was changed, the culture was shifted to the next mode; each successive mode was designated by a Roman numeral.
[b]HiGg media were balanced with respect to C, N and P when the gravimetric ratio of assimilable C, N and P was 100 : 9-10 : 1, as in mode 1 of culture N. Other ratios imposed specific limitation of growth by C (eg 48 : 9 : 1 in N/VI), by N (eg 101 : 6.6 : 1 in N/II), or by P (eg 194 : 38 : 1 in N/V).

Table 2 Duration and productivity per mode in *C. crescentus* perpetual cultures

Culture mode	Hours	No. Gen.[a]	Per ml of culture:				Productivity rates	
			10^9 CFU	μg DW	xs μg glc	xs μg Pi-P	μg DW h^{-1}	10^9 CFU h^{-1}
J/I	0–96	11.5	3.7	320	15	<0.01	9.0	104
J/II	97–192	11.5	4.3	499	19	<0.01	14.0	120
J/III	193–288	11.5	4.6	671	23	<0.01	18.8	129
J/IV	289–372	10.1	4.6	1162	46	<0.01	32.5	129
J/V	373–576	24.5	1.7	396	18	<0.01	11.1	48
K/I	0–71	8.5	1.7	356	53	<0.01	10.0	48
K/II	72–167	11.5	1.7	517	116	<0.01	14.5	48
N/I	0–98	11.8	6.0–1.6	905–598[b]	108–507	<0.03	15–17	168–45
N/II	99–266	20.1	4.9	496	277	0.39	13.9	137
N/III	267–312	5.4	5.7–0.7	671–394	(794)[c]	(0.56)	19–11	160–20
N/IVa[d]	313–435	14.7	4.4	499	30	1.06	14.0	123
N/IVb[d]	436–504	8.2	5.7	575	14	1.14	16.1	160
N/V	505–696	23.0	3.6	591	34	<0.06	16.6	101
N/VI	697–866	20.4	5.7	585	16	1.13	16.4	160
C/I	0–240	28.9	2.7	558			15.6	76
C/IIa[e]	0–189	22.6	3.6	503			14.1	101
C/IIb[e]	190–649	55.4	3.6	542			15.2	101
C/III	650–980	39.8	3.8	1080			30.2	106

[a]No. of generations was calculated as = t/t_h, elapsed time divided by hydraulic half-time. In these cultures, t_h was 8.3 h.
[b]For non-steady states and for otherwise steady states during which substrate uptake rates changed steadily in one direction, two values are given; the first is the first rate determined, and the second is the last determination during that mode.
[c]Values in parentheses represent determinations with a single culture sample during the mode. All other values are averages of determinations with samples removed from the culture at least three times during the mode.
[d]See Figure 4.
[e]Perpetuation of culture C required replacement of the reservoir during mode II; although intended to be identical in composition, clearly there was some difference between the two batches of reservoir medium prepared for mode II.

Sampling from perpetual cultures

Samples were withdrawn directly from the culture through the sampler on the BioFlo culture vessel and chilled immediately by swirling in an ice-water slush. The volume removed at any time depended on the number of assays to be performed. The smallest samples, for determinations of turbidity, pH and viable counts only, were approximately 5 ml. The largest samples, required for segregation of stalked and non-stalked cells, were 100 ml. After removal of such a large sample, flow was accelerated in order to hasten return of the culture to its previous volume, and at least two volume changes were allowed before the next sampling.

Viable counts

Viable counts were determined from two parallel dilution series in half-strength peptone/yeast extract broth prepared from each culture sample. An aliquot of appropriate dilution was placed on the agar surface, then spread over the agar by the addition of 2 ml of molten soft agar. The plate was incubated at 30°C, and colonies were counted after 3 days of incubation. Four aliquots were plated from each dilution series so that each viable count was based on colony counts from eight plates. Routinely, plating error was within ± 5%. For cultures J, K and N, microscopic counts were also performed in a Petroff-Hausser chamber; because these counts were not significantly different from the viable counts, only viable counts are reported.

Assays of pH and of residual nutrients

A chilled sample of culture of at least 5 ml was centrifuged at 27000 × *g* at 4°C and the clear supernatant phase was aspirated to a plastic, screw-capped tube. The pH of a sample of the supernatant warmed to room temperature was determined, and the remainder of the supernatant was stored frozen. Thawed supernatants were used for assays of residual glucose by the anthrone reaction [61] and phosphate by the molybdate 'kit' procedure [35].

Assays of dry weight, protein, nucleic acids and reserve materials.

Turbidity was determined in a Klett-Summersen colorimeter calibrated to convert Klett units (KU) to dry weight density through calibration curves prepared for large (PHB-laden) cells and for small (low-PHB) cells, and for segregated stalked and non-stalked cell populations. Each of these populations scatters light as a linear function of density, but the slopes of turbidity as a function of density vary. Protein was determined on culture samples, washed cells prepared for substrate uptake assays, and on washed and frozen cell pellets by the method of Lowry *et al* [29] after solubilization of proteins by heating with 1 N NaOH for 30 min; bovine serum albumin was used as protein standard. RNA and DNA were assayed by the orcinol (total pentose) and diphenylamine (deoxyribose) procedures [51]. RNA varied from 15 to 20% of protein content; DNA varied from 2.9 to 5.9 μg per 10^9 CFU, reaching its minimum in culture K, mode II, where the proportion of dividing cells (7% of the population) was the lowest observed.

Reserve materials were assayed using frozen cell pellets. PHB was determined turbidimetrically in alkaline hypochlorite reagent, and polyphosphate by molybdate reaction of phosphate freed by acid hydrolysis as described previously [45].

Segregation of stalked and non-stalked cells

To segregate stalked and non-stalked cells by differential centrifugation [56], a chilled 100-ml sample of culture was centrifuged at 8000 × *g* for 10 min at 4°C. The supernatant phase and fluffy layer of cells were decanted and centrifuged at 20000 × *g* for 15 min. The clear supernatant phase was aspirated and discarded, then the loosely-packed cells were resuspended in Hi basal medium, centrifugation was repeated at 20000 × *g*, and the loosely-packed cells finally resuspended in Hi basal medium. The first pellet of cells that packed densely at 8000 × *g* was resuspended in Hi basal medium, recentrifuged at 8000 × *g*, and finally resuspended in Hi basal medium. The suspension of densely-packing cells comprised >95% non-stalked cells, and the suspension of loosely-packing cells comprised *ca* 99% stalked cells; each population had been washed once in Hi basal medium.

Measurements of cell dimensions

To prepare cells as shadowed specimens for examination by electron microscopy, 1 ml of culture was diluted with 9 ml of glass-distilled water (GDW), centrifuged at 27000 × *g*, then resuspended in 1 ml GDW and immediately placed on nitrocellulose-coated copper grids. After 3–5 min, the fluid was gently drained from the grid and the specimen allowed to dry in air. Dried specimens were shadowed with Pt : Pd, 80 : 20, and examined in a Philips EM300 operated at 60 kV. Electron micrographs were printed at 5–10000× magnification, and cell and stalk lengths were measured with a Numonics integrating planimeter. Length measurements shown below are averages for 200–400 cells and 100–300 stalks per mode for each culture except mode V of culture J, from which only 31 cells and 23 stalks were measured.

To determine surface area per cell, excess cells from the cell segregation procedures during cultures J and K were repooled and their peptidoglycan sacculi were purified by solubilization and washing away of all other cell components. The purified sacculi were laid flat on nitrocellulose-coated copper EM grids, stained with uranyl acetate, and rotary shadowed at a low (<10°) angle, as described previously [46,47]. Electron micrographs were printed at 10800× magnification, and the area of each sacculus was measured with a Numonics integrating planimeter. The complete surface area was calculated as twice the measured area; because the sacculus is only 4 nm thick, the minute fraction of surface at the edge of each sacculus was disregarded. The areas of 70–80 sacculi were measured for each pooled population. The length of each 'stalkulus' (the peptidoglycan skeleton of the stalk) was also recorded; the transverse dimension of the flattened stalkulus was constant at 92.6 nm; twice this value was regarded as the circumference, and the stalkulus surface area was calculated as 2 × 0.0926 μm × length.

Alkaline phosphatase assay

Alkaline phosphatase was assayed according to the method of Tommassen and Lugtenberg [59] employing Sigma 104 Phosphatase Substrate (*p*-nitrophenylphosphate, 'pNPP'). A sample of culture was mixed with 1/20 volume of toluene for 60 s at room temperature, then a sample of the toluenized cells was transferred to 0.5 ml of pNPP reagent in Tris buffer, 0.1 M, pH 10.0, prewarmed to 30°C. Reaction was stopped after 20 min by the addition of 0.5 ml 1 N NaOH and absorbance was read at 410 nm in a Zeiss PMQ spectrophotometer.

Substrate uptake assays

A chilled sample of culture was centrifuged at 27000 × *g* for 15 min, washed once by resuspension in Hi basal medium and recentrifugation, then finally resuspended to a turbidity of 100 Klett units (KU) (*ca* 10^9 CFU ml^{-1}). Samples were removed for determinations of protein and viable count, then the suspension was either used directly or diluted to 50 or 25 KU for determination of uptake rate. With populations containing cells with very long stalks, it was necessary to use more dilute suspensions; the long stalks retarded filtration and washing, allowing continued substrate uptake after the timed interval, a problem eliminated by the use of low-density suspensions. To determine the initial rate of uptake, a small volume (70–105 μl) of suspension was warmed briefly to room temperature, carrier or diluent was added, and then radiolabeled substrate was added. After 15–30 s, a 50 μl sample was removed to a membrane filter mounted on a Millipore vacuum manifold so that liquid moved promptly through the filter, which was immediately washed with three 2-ml aliquots of distilled water. The membrane filter was removed to paper toweling to dry, then placed in a scintillation vial; 5 ml of Ultraflor scintillation fluid was added and the counts were determined in a Beckman LS65 liquid scintillation counter on the day of assay. Radiolabeled substrates were obtained from New England Nuclear, Boston, MA, USA. Phosphate was provided as ^{32}P-phosphate, ^{3}H-amino acid(s) either as leucine (for cells from cultures J, K and C) or as a mixture of amino acids (for cells from culture N), and ^{3}H-glucose as D-glucose. For comparative purposes, all values shown here are calculated for 1 μM substrate concentration. However, each rate was routinely measured at several substrate concentrations, ranging from <1 nM to 1 mM; for some populations, uptake (particularly of leucine) was saturated at 1 μM substrate.

Glucose was added to 0.2% (wt/vol) to the suspending medium for suspensions from cultures J and K. However, the presence of glucose did not influence rates of uptake of phosphate or leucine. Glucose was not added to assay suspensions for cultures N and C, allowing glucose uptake to be measured with and without carrier glucose.

Chemotaxis assay

The assay was a version of the microcapillary assay method of Adler [1], modified for strict aerobes whose locomotion ceases within a few minutes under a cover slip when the suspension mounted exceeds about 5 × 10^8 cells ml^{-1}. Instead of the U tube-closed chamber of Adler's design, 0.2 ml of bacterial suspension was loaded into a shallow depression slide with a cover slip over one-half the width of the depression; the vertical meniscus provided a relatively large surface for the diffusion of air into the suspension. The test solution that generated the solute gradient in the chamber was loaded into an unsealed 1-μl capillary tube ('microcap') by allowing the microcap to stand (on end 'A') for about 1 s in 2 ml of solution in a 10-ml beaker.

To begin an assay with cells from culture N, 5 ml of culture was centrifuged at 8000 × *g* for 10 min, and the supernatant and loosely-packed cells (stalked, non-motile) were discarded. The swarmer-enriched pellet was gently resuspended in 10 ml of Hi basal and centrifugation was repeated. The second pellet was resuspended and diluted to approximately 10^7 cells ml^{-1} in aerated diluent and used promptly for the assay. Cells from culture C were not washed; the culture was diluted into aerated Milli-Q filtered distilled water as needed to achieve 10^7 cells ml^{-1} (at least 150-fold). As soon as possible after the cell suspension was delivered to a depression chamber, two microcaps were loaded with diluent or test solution, rolled on filter paper to blot any liquid adhering to their outer surfaces, and end 'B' of each was introduced into the suspension. At the end of the incubation time (routinely 20 min), each microcap was removed, rolled on paper to blot its outer surface, then its contents were expelled into 1.0 ml of diluent through end 'A'. At least two aliquots of the diluted microcap contents were transferred to the surface of agar medium and each was spread by the addition of 2 ml of molten soft agar medium at 50°C. Plates were incubated at 30°C, and colonies were counted after 3 days of incubation.

Routinely, an assay included one chamber per test solution per concentration, two microcaps per chamber, and two aliquots were plated from each microcap, so that each count of CFU per microcap was based on counts of colonies on four plates. Controls were duplicated and so were based on counts of colonies on eight plates. Diluent-only microcapillaries typically contained 1000–5000 CFU.

Stock solutions of test substances were prepared using reagent grade chemicals, filter-sterilized, and stored refrigerated; dilutions were prepared in Hi basal medium for assays with cells from culture N and in Milli-Q filtered water for cells from culture C.

Results

Four perpetual cultures were studied. The culture parameters are given in Table 2, cell composition in Table 3, and cell dimensions in Table 4. Some characteristics of cultures J and K were displayed in a previous report [43].

Phosphorus limitation

The first two cultures (J and K) were established to characterize responses to increasing severity of phosphorus (P) limitation relative to the supply of carbon (C) and nitrogen (N) by periodically adding glucose and glutamate to the reservoir medium (Table 1; J, modes I–IV) while NH_4Cl and phosphate concentrations remained constant. This sequence was followed by a three-fold decrease in each of glucose, glutamate and phosphate concentrations to change their absolute, but not their relative fluxes during mode V. The second culture (K) was initiated to replicate mode V

Table 3 Cell composition

A. All cells

Culture/mode	μg per 10^9 CFU				APase nmol min^{-1} mg^{-1} protein
	dry weight	protein	PHB	polyP-P	
J/I	87	62	<4.0	0.16	79
J/II	116	75	13.7	n.d.[a]	111–265
J/III	146	94	28.0	n.d.	239
J/IV	253	110	80.4	n.d.	275
J/V	233	98	69.4	n.d.	275
K/I	209	98	60.0	n.d.	175
K/II	304	107	109.4	n.d.	239
N/I	139–374[b]	81–243	30–46	0.05–0.17	
N/II	101	62	6.7	0.065	
(N/III)	118–563	(479)[c]	(34)	(0.82)	
N/IVa[d]	113	85	3.0	0.079	
N/IVb[d]	101	68	2.3	0.050	
N/V	164	91	26.7	0.017	
N/VI	102	79	2.6	0.059	
C/I	207				
C/IIa[e]	140				
C/IIb[e]	151				
C/III	284				

B. Segregated Populations

	μg dry wt per 10^9 CFU		μg protein per 10^9 CFU		APase, nmol min^{-1} mg^{-1} protein	
	S cells	N cells	S cells	N cells	S cells	N cells
J/I, 72 h	83	66	53	40	85	89
J/II, 168 h	126	105	69	55	240	235
J/III, 266 h	189	129	101	64	295	243
J/IV, 360 h	251	180	119	73	241	206
K/I, 48 h	217	183	177	123	139	159
K/II, 167 h	396	273	304	188	174	194
N/II, 264 h	108	93	70	50	–	–
N/IV, 435 h	105	83	80	57	–	–
N/V, 696 h	225	157	144	83	–	–
N/VI, 866 h	139	95	105	61	–	–

[a]Not detected; limit of detection was 0.05 μg Pn-P ml^{-1} culture.
[b]For non-steady states and for otherwise steady states during which substrate uptake rates changed constantly in one direction, two values are given; the first is the first rate determined, and the second is the last determination during that mode.
[c]Values in parentheses represent determinations with a single culture sample during the mode. All other values are averages of determinations with samples removed from the culture at least three times during the mode.
[d]See Figure 4.
[e]Perpetuation of culture C required replacement of the reservoir during mode II; although intended to be identical in composition, clearly there was some difference between the two batches of reservoir medium prepared for mode II.

of culture J, then once again the fluxes of glucose and glutamate were increased without increasing the flux of phosphate (or of NH_4Cl) in order to determine whether responses to increased severity of P limitation would be affected by the lower absolute fluxes of C, N and P.

Culture J was provided with a medium with a C : N : P ratio of 77 : 36 : 1 and should have been C-limited in mode I. It was expected that C-limited cells would express constitutive levels of properties and activities related to P acquisition and storage, maximal consumption and minimal storage of C, and development of stalks of moderate length. Shifting the nutrient flux from P-excess and C-limitation to C-excess and P-limitation was expected to result in corresponding shifts in cellular composition, activities and morphogenesis.

In most respects, these expectations were realized with culture J (Tables 2, 3, 4 and 5). As the nutrient flux changed from C-limitation through three successively more severe P-limitations, PHB storage increased, and the cells became more massive; protein content decreased as a proportion of dry weight as the proportion accounted for by PHB increased. The rate of phosphate uptake increased (by every comparison: per cell, per unit dry weight or protein, and per unit volume of culture), the specific activity of alkaline phosphatase increased, and cell and stalk lengths increased as a direct function of the increased C : P ratio in the medium (Figure 1).

Table 4 Cell dimensions

	Cell length, μm			Stalk length, μm		Area[a], μm^2	
	N cell	S cell	Div cell[b]	All	3-Band[c]	N cell	S body + Stalk = Total
J/I	1.4	1.7	2.5	1.4	2.1	0.724	1.146 + 0.264 = 1.410
J/II	1.5	1.8	2.6	1.9	2.9	0.711	1.046 + 0.356 = 1.402
J/III	1.7	2.1	3.0	3.1	4.0	0.853	1.178 + 0.574 = 1.752
J/IV	1.8	2.4	3.4	3.9	4.8	1.181	1.587 + 0.712 = 2.299
J/V	1.4	2.2	2.6	4.4	(4.4)		
K/I	1.7	2.3	2.9	3.6	4.1	1.090	1.355 + 0.858 = 2.213
K/II	1.9	2.6	3.8	5.4	5.8	1.446	1.661 + 0.982 = 2.643

[a]Area was determined by measurement of electron micrographs of peptidoglycan sacculi.
[b]Division by constriction is accompanied by transient cell elongation as the constriction develops into the taper of the new cell poles.
[c]Three bands are present only after the cell has divided at least once. Comparison of 3-banded stalks provides a comparison of stalks of the same age in each population.

Table 5 Substrate uptake by steady-state populations

Culture/mode	pmol min^{-1}					
	per 10^9 CFU			per mg protein[a]		
	phosphate	amino acid	glucose	phosphate	amino acid	glucose
J/I	25–17[b]	60	–	377–263	942	–
J/II	39–77	54	–	520–1041	719	–
J/III	106	57	–	1166	623	–
J/IV	193	73–59	–	1776	629–532	–
J/V	(359)[c]	(65)	–	(4128)	(745)	–
K/I	73	27	–	854	278	–
K/II	151	14	–	1096	100	–
N/I	45	118–14	6	300	959–68	36
N/II	4	193	7	65	3399	60
N/III	(10)	(433)	(12)	(26)	(1098)	(31)
N/IVa[d]	4	max 976	6	75–42	max 12531	56
N/IVb[d]	2	min 133	max 21	28	min 1913	max 309
N/V	131	94–293	11	1168	900–2125	91
N/VI	10	88	60	127	1118	765
C/I	10	64–243	9	79	508–1929	71
C/IIa[e]	16	89–135	8	188	1047–1588	94
C/IIb[e]	16	576	1	174	6261	11
C/III	170	24	5	983	139	29

[a]Protein content estimated for culture C populations.
[b]For non-steady states and for otherwise steady states during which substrate uptake rates changed constantly in one direction, two values are given: the first is the first rate determined, and the second is the last determination during that mode.
[c]Values in parentheses represent determinations with a single culture sample during the mode. All other values are averages of determinations with samples removed from the culture at least three times during the mode.
[d]See Figure 4.
[e]Perpetuation of culture C required replacement of the reservoir during mode II; although intended to be identical in composition, clearly there was some difference between the two batches of reservoir medium prepared for mode II.

Meanwhile, polyphosphate storage ceased when the C : P ratio exceeded 100 : 1 (mode II and following), and although absolute glucose consumption increased per unit time, a progressively higher concentration of glucose remained unused in the culture medium. Decreasing the absolute fluxes of glucose, glutamate and phosphate at the same ratio for mode V reduced population density (mass and CFU) and residual glucose, each by about three-fold, but did not alter cell composition (Table 3). Culture J was interrupted after only one sample from mode V had been assayed for substrate uptake rates, which seemed to have increased (Table 5). Culture K, mode I, provided cells of approximately the same size and general composition as in J/V, but specific activity of alkaline phosphatase and rates of phosphate and leucine uptake were significantly lower. Nevertheless, the responses to increased C : P flux (K/II relative to K/I) were the same as seen throughout culture J with regard to every parameter employed.

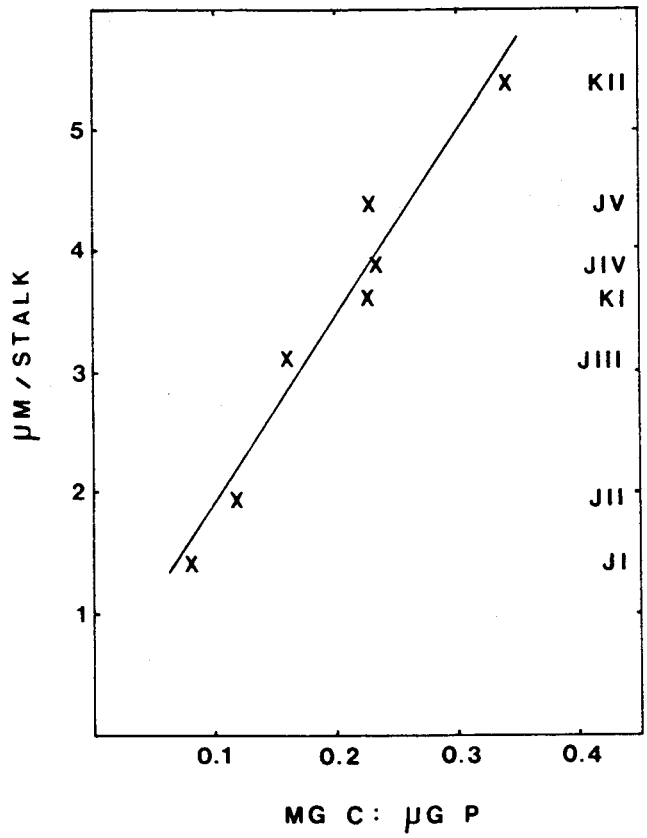

Figure 1 Influence of the ratio of carbon to phosphorus in the culture medium on average stalk length. The culture and mode corresponding to each point are indicated along the right border.

As usual in healthy, reproducing populations of *C. crescentus*, three types of cells were present: dividing cells that bore a stalk at one pole and (often) a single flagellum at the other, plus the two types of unconstricted cells that arose from completion of cell division: non-motile stalked cells and non-stalked swarmer cells. In P-limited populations dividing every 8.3 h, constricted cells accounted for not more than 10–11% of the population.

In order to determine whether the various changes recorded for each total population occurred equivalently in the stalked and non-stalked cells, one sample of each of six steady-state populations (J/I – IV and K/I and II) was segregated to provide suspensions of N cells (non-stalked, flagellation not determined) and of S cells (stalked, with and without constrictions).

The results, shown in Tables 3, 4 and 6, revealed that the principal difference between the S and the N cells was the greater size of the S cells, whether measured as cell body length (cell diameter was the same), or as mass or protein per cell. Accordingly, it was not surprising that physiologic activities calculated on a per cell basis resulted in larger values for S cells than for N cells. The activities of the phosphate-regulated enzyme alkaline phosphatase were not significantly different for S cells and N cells when calculated per unit of protein. The stalked and non-stalked stages of the cell cycle appeared comparably derepressed for alkaline phosphatase activity by P fluxes that imposed P-limitation on cell activities.

In contrast to alkaline phosphatase activity, the rate of phosphate uptake was considerably higher in S cells than in N cells, whether calculated per cell or per unit protein. This uptake activity appeared comparable in the two cell types only when calculated as activity per unit of cell surface area, and only when the surface area of a stalked cell included the surface of the stalk as well as the surface of the cell body (Table 6). These results revealed that uptake per cell was a function—topologically, as well as physiologically—of total surface area per cell. S cells took up phosphate faster because their surface area was greater, due both to their greater length, which increased the surface : volume ratio relative to that of N cells, and to the presence of stalks, which added surface to each cell without increasing its cytoplasmic volume. Although uptake activity has been demonstrated in stalks sheared from *Asticcacaulis biprosthecum* cells [27,49], this is the first direct experimental evidence of participation of the stalk surface of intact caulobacterial cells in nutrient uptake.

Leucine uptake was included in this study of phosphate limitation to provide a measure of cell surface activity that might be indifferent to P flux. Unexpectedly, the rate of leucine uptake decreased as relative P flux decreased. However, what is planned is not necessarily what is executed, and the decrease in leucine uptake through this series of P-limitations was more reasonably attributed to the changing ratio of N sources to each other (next section).

Nitrogen limitation

The third culture (N) was to be challenged to respond to shifting identity of the limiting nutrient flux among C, N and P sources, while minimal change in population density was allowed. This culture experienced two periods of intolerable relative fluxes, in modes I and III, during which the populations declined (Figure 2) and cell morphology became bizarre and irregular. These two unsteady states were nevertheless instructive with regard to both morphogenesis and the oligotrophic physiology of *C. crescentus*.

The medium for mode I (0 to 98 h) provided a C : N : P ratio of 100 : 9 : 1, and it was expected to provide nutritional balance among these three elements. The media used in our previous perpetual cultures (J and K; see also [38,45]) had provided 55% of the C as glucose and 45% as glutamate. However, in order to simplify calculations for the shifts in nutrient ratios planned for culture N, 88% of the C was provided in the form of glucose and only 22% as glutamate, while NH_4Cl provided the remainder of N for the ratio of 9 μg total N per μg P. This had dramatic effects on morphogenesis, reproduction, and substrate consumption.

Within 12 h of initiation of medium flow to the culture, cell diameter had increased noticeably. As flow continued, the cell poles became blunt, at most only stubby stalks developed, motility decreased, and pre-division constrictions were placed irregularly along the cells, resulting in wide variation in cell length. Granules of PHB became large and refractile and were packed within the cells. The viable count declined, but not so rapidly as would have occurred if reproduction had ceased. The reproductive rate could be calculated, as mean cell cycle time (t_c), from the relationship between the increase in cell number that would

Table 6 Substrate uptake by segregated populations

Culture/mode	Cells	pmol Pi min^{-1} per:			pmol leu min^{-1} per:		
		10^9 CFU	mg protein	μm^2 surface body + stalk	10^9 CFU	mg protein	μm^2 surface body + stalk
J/I, 72 h	S	20	375	17 14	90	1694	79 64
	N	14	360	19	52	1307	72
J/II, 168 h	S	78	1147	75 56	80	1181	76 57
	N	47	859	66	37	674	52
J/III, 266 h	S	153	1552	130 87	69	704	59 39
	N	68	1063	80	35	550	41
J/IV, 360 h	S	216	1828	136 94	53	450	33 23
	N	97	1333	82	26	357	22
K/I, 48 h	S	116	638	86 52	41	228	30 19
	N	58	476	53	21	174	19
K/II, 167 h	S	291	989	175 110	23	76	14 9
	N	169	916	117	10	55	7

		pmol Pi min^{-1} per:		pmol leu min^{-1} per:		pmol glc min^{-1} per:	
		10^9 CFU	mg protein	10^9 CFU	mg protein	10^9 CFU	mg protein
N/II, 264 h	S	4.0	61	256	3817	2.3	35
	N	1.9	38	201	3989	2.3	46
N/IV, 435 h	S	2.6	33	347	4324	11.7	145
	N	2.3	40	236	4054	4.6	80
N/V, 696 h	S	213	1433	319	2143	16.4	111
	N	72	884	237	2917	6.3	77
N/VI, 866 h	S	26	245	133	1274	159	1525
	N	16	263	91	1474	77	1247

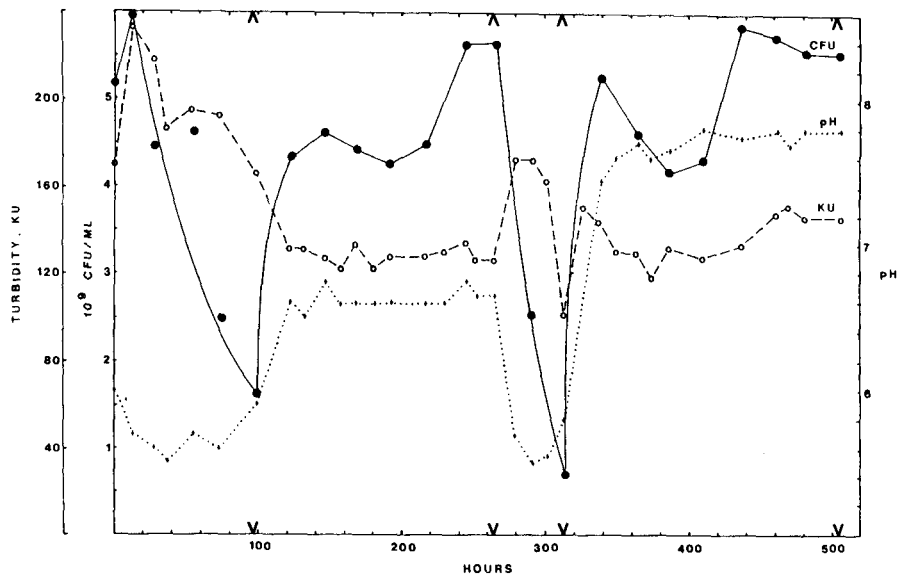

Figure 2 Declines and recoveries of perpetual culture N. (•) 10^9 Colony-forming units ml^{-1} culture. (○) Turbidity, as Klett units. (+) pH. Arrow heads along top and bottom borders indicate the times at which media were changed (98 h, 266 h, 312 h, 504 h).

result from binary fission every $t = t_c$ (proportional increase $= N \times 2^{t/t_c}$), and the decrease in cell number that would result from liquid flow through the culture every $t = t_h$ (proportional decrease $= N \times 2^{t/t_h}$), as:

$$N_t = N_o\,(2^{t/t_c})(2^{-t/t_h}),$$

where t_h is the hydraulic half-time of the system (equal to

R ln2), and t is elapsed time during which viable count changed from N_o to N_t. Solving for mean cell cycle time:

$$[(\ln N_t/N_o)/(t \cdot \ln 2)] + 1/t_h = 1/t_c,$$

and using the viable counts determined at four points during mode I, the reproductive rate of the population was calculated as 10.4 h per generation. This t_c was longer than t_h, as occurs during a decline in density of a viable population [22]. (See Figure 2. The count at 54 h was aberrant; although no technical error could be found to account for its divergence, it did not fit the otherwise clearly exponential decline and was not included in calculation of t_c.)

Although phosphate consumption continued efficiently, the concentration of residual glucose increased steadily. Dry weight, protein, PHB and polyphosphate per cell increased as the cells enlarged, divided infrequently, and stored both C and P. Uptake rates per unit protein were at constitutive levels for glucose and phosphate, and although amino acid uptake began at a constitutive level, it fell to the lowest specific rate observed throughout this study (Table 5). Also during this mode, the pH had dropped to less than 6.0 (Figure 2); in the 14 steady states reported here, culture pH was 6.6 during stable N-limitation, and 7.7–7.8 during stable C- or P-limitation.

In hindsight, the cells appeared to be unable to utilize ammonium as the principal source of N in this medium, an interpretation substantiated by swift recovery (Figure 2) of the culture in mode II (99–266 h), initiated by reduction of the NH_4Cl concentration in the reservoir medium. This was not done because the situation was understood at the time, but in order to proceed from an unexplained failure to attain a C/N/P balanced steady state to a N-limited state. Within 24 h, morphology was returning to normal (small cells of regular length, long stalks, lively motility, cell poles tapered, regular division sites, etc), and viable count had risen without an increase in turbidity. The rate at which the viable count was restored required a cell cycle time of not more than 5.5 h (for calculation, see above), so that cells were produced faster than hydraulic flow removed them. Only binary fission was detected by microscopical examination; the large cells did not constrict at more than one site at a time within any one cell.

The cause of the condition of the culture in mode I still not being appreciated, all these troubles were revisited on the culture when both NH_4Cl and phosphate were added to the reservoir medium to change the nutrient ratio to 66 : 10 : 1, intended to impose C-limitation for mode III (267–312 h) without reducing the C flux. Instead, the changes seen in mode I recurred, more severely and more rapidly. During the mode III decline, the average t_c was 17.5 h and motility was abolished entirely. The cells were recognizable as caulobacters only by the occasional presence of a typical stalk, complete with bands, on an otherwise monstrous cell (Figure 3).

Normal morphology and population density were restored by reducing the reservoir concentrations of both glucose and NH_4Cl, and by providing the rest of the C and N in the form of glutamate. The phosphate concentration was changed (increased) only slightly. The C : N : P ratio of the medium for mode IV was 48 : 9 : 1, with one glutamate-N per ammonium-N; this should have imposed C-limitation.

Recovery of morphogenesis, motility, and reproduction was prompt. In contrast to mode II, recovery in mode IV was accompanied by an increase in turbidity. To account for the rise in viable count observed within 12 h of the initiation of mode IV, reproduction must have occurred with a cell cycle time not longer than 4.1 h, and morphology was normal through at least 90% of the population by the end of the third generation. Adjustment of substrate uptake activities, however, was not prompt (Figure 4). For about 12 generations (mode 'IVa'), the culture exhibited an exceptionally high capacity for amino acid uptake–the highest observed for culture N, and comparable to that observed in mode II of culture C, where the lowest proportion of N was provided (see Tables 1 and 5). As the capacity for amino acid uptake began to drop to constitutive levels, a significant increase in glucose uptake rate began (mode 'IVb'), accompanied by a 30% increase in CFU ml^{-1} and a 15% increase in turbidity (Figure 2). These somewhat smaller cells continued to increase in capacity for glucose uptake; a maximum glucose uptake rate was not achieved by the time the culture was shifted to P-limitation (mode V).

Because *C. crescentus* lacks glutamic dehydrogenase and is dependent upon an ammonium-regulated GS/GOGAT system for ammonium assimilation [13], the experiences of this perpetual culture were interpretable as due to extreme N-starvation resulting from the high ratio of ammonium-N to glutamate-N. In all subsequent attempts to impose N-limitation, media provided at least 1 μg glutamate-N per μg ammonium-N. Wide variations in relative flux of both glucose and of phosphate are tolerated by perpetual cultures provided with such media, in which glutamate and ammonia together provide assimilable N.

During incubation of culture N, cells were periodically withdrawn from the culture and used as inocula for batch cultures in the reservoir media, unsupplemented and supplemented with glucose, NH_4Cl, phosphate, or all three nutrients. Cells from 120 h were inoculated into reservoir media I and II, cells from 359 h into media III and IV, and cells from 815 h into all six media (I–VI). The purposes of this secondary cultivation were to determine: (a) the rate of exponential growth that each medium would support; (b) the identity of the supplementing nutrient that would increase yield in a batch culture, since that is the nutrient that would limit population density and productivity in the perpetual culture; and (c) whether the population's ability to grow in these media changed during perpetual cultivation.

Five of the unsupplemented media supported exponential growth at a specific rate of 0.214 h^{-1} (3.2 h/doubling); growth was somewhat slower in reservoir medium V (the low-P medium), occurring at a specific rate of 0.193 h^{-1} (3.6 h/doubling). All yields in the unsupplemented media were higher than in the perpetual culture (see Table 2); batch culture yields, as μg dry wt ml^{-1}, were: 800 in medium I, 585 in medium II, 702 in medium III, 624 in media IV and VI, and 630 in medium V. Yield was increased by NH_4Cl supplementation of media I and II, and

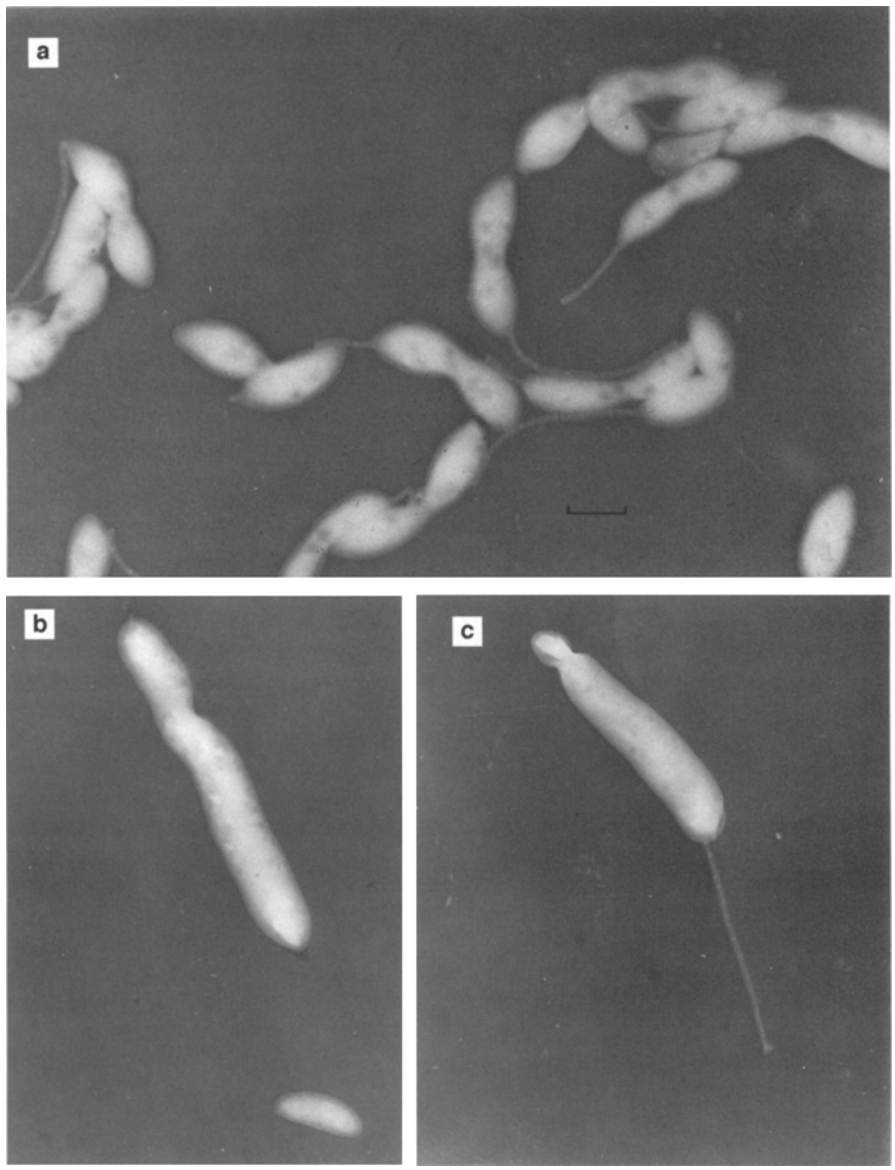

Figure 3 Appearance of cells during mode III of culture N. (a) early cells (290 h) appeared swollen, but not misshapen. (b, c) Late cells (312 h) were wider, irregular in outline, and usually divided abnormally, although an occasional cell appeared unaffected (the small vibrioid swarmer in (b). Shadowed electron micrographs, negative image; the marker is 1 μm and applies to all three micrographs.

by glucose supplementation of media IV and VI. Glucose supplementation of medium V enhanced dry weight yield, all accounted for by PHB. None of the supplementations enhanced yield in medium III; unfortunately, glutamate supplementation was not tested. Thus, the batch cultures were yield-limited by nitrogen (I and II), by carbon (IV and VI), and by phosphorus (V); glutamate probably limited yield of the batch cultures in medium III. Rates, yields and lag times (in every case not more than 3 h) were not different for 120-h, 359-h and 815-h cells.

The course of events in culture N implied an explanation for the decline in rate of leucine uptake in the successive modes of culture J. In that culture, successive additions of glucose and glutamate, intended to decrease the relative P flux, simultaneously decreased the ratio of ammonium-N to glutamate-N. As shown in Figure 5, the progressive decrease in this latter ratio was directly correlated with decreased rate of leucine uptake. (Leucine uptake correlated equally well with ammonium flux per unit dry weight in the culture because dry weight was a function of the reservoir concentrations of the C-sources, glucose and glutamate together.) Apparently, when ammonium flux exceeded availability of the ammonium acceptor, glutamate, the unassimilated ammonium reduced the capacity of the cells to assimilate that ammonium. The cells were N-deprived and, in response, increased their uptake capacity for organic N; progressively, as more acceptor became available, the response became less intense.

Successive nitrogen, phosphorus, and carbon limitations

A major goal of studies with culture N was to determine whether cells would become chemotactically responsive to

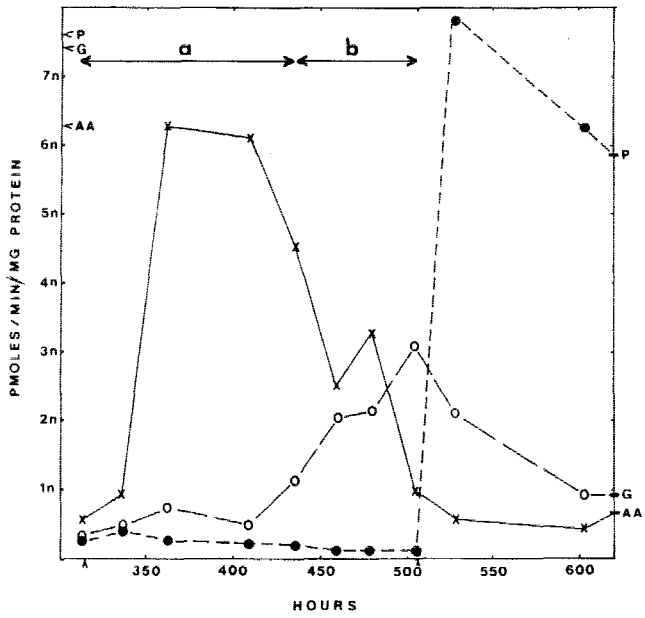

Figure 4 Substrate uptake rates in mode IV of culture N. (x) Amino acids (AA; $n = 2000$). (○) Glucose (G; $n = 100$). (•) Phosphate (P; $n = 200$). Arrowheads at the left border indicate maximum uptake rate observed at some point during culture N; the short bars in the right border indicate the activities that persisted through mode V. Mode IV began at 313 h and ended at 504 h (small arrowheads along bottom border); modes 'IVa' and 'IVb' are indicated by the horizontal arrows.

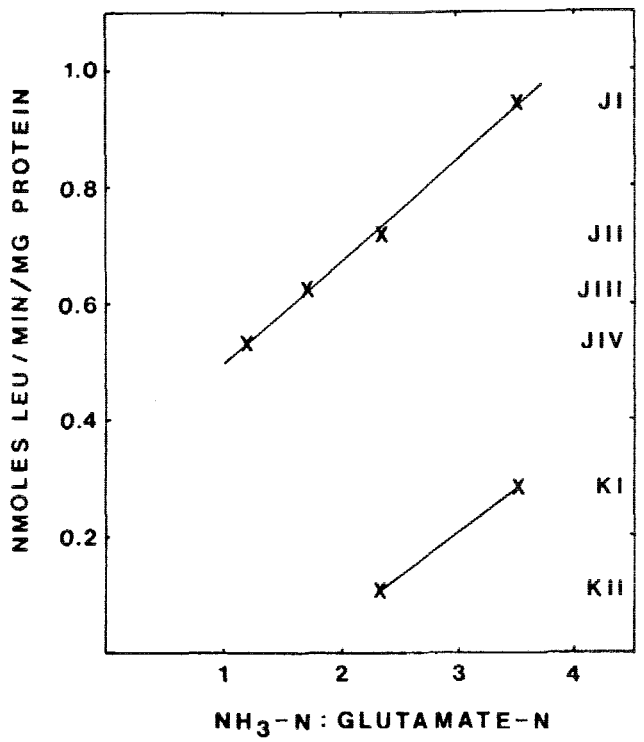

Figure 5 Influence of ratio of nitrogen sources on rate of leucine uptake by steady-state populations in culture J (upper line) and culture K (lower line). The culture and mode corresponding to each point are indicated along the right border.

gradients of solutes emerging from microcapillaries when cultivated under conditions of nutrient limitation and, if so, whether responses would be elicited specifically by gradients of the limiting nutrient. As judged from substrate concentrations in culture supernatants (Table 2), PHB and polyphosphate content of the cells (Table 3), and rates of uptake of amino acids, phosphate, or glucose (Table 5), three steady states were achieved in which the populations appeared limited specifically for N (mode II), for P (mode V), or for C (mode VI). A subsequent culture (culture C) was intended to approximate mode II (N-limited) of culture N (but with only 1 μg ammonium-N per μg glutamate-N), then to increase the severity of N-limitation, gently, by increasing the fluxes of glucose and phosphate, and finally to relieve N-limitation by adding glutamate and NH_4Cl to the medium and reducing the phosphate concentration to ensure that the culture was shifted to P-limitation.

Results of the microcapillary assays for chemotaxis are presented in Table 7. Responses interpreted as positive ranged from 1.3 to almost 10 times as many cells per microcapillary if it contained an attractive solute rather than diluent alone. This is a very weak response, numerically; increasing the incubation time of the microcapillaries did not increase the ratios, and often reduced them. Nevertheless, the overall result of both experiments was repeatably detectable responsiveness, but only to gradients of potential N sources. Swarmers that arose during N-limited growth (N/II and C/II) or recovery from it (N/IV) responded to gradients of methionine, NH_4Cl and glutamate. Responsiveness to NH_4Cl gradients continued during subsequent P-limitation in culture N (mode V). At one sampling time (833 h), the swarmers of mode VI seemed somewhat responsive to gradients of glucose and phosphate, but after prolonged C-limitation, responsiveness was not detected in gradients of any solute tested.

Modes IV and VI of culture N were provided with the same medium (Table 1). However, for about 12 generations of mode IV (mode 'IVa'), the population was recovering from the adverse effects of the high ammonium : glutamate medium of mode III. During mode IV, responsiveness was more apparent in mode 'IVa' (361 h; 5.8 generations), while the cells still behaved as though limited for N, than in mode 'IVb' (459 h; 17.6 generations), when the cells were adjusting to C-limitation. In contrast to events in mode IV, as the culture entered mode VI after 23 generations of P-limitation, a C-limited steady state was established within 71 h (8.6 generations); chemotactic responsiveness to amino acids was not detectable, and responsiveness to NH_4Cl was eventually lost.

Summary of results

Eight potential responses to nutrient limitation were assessed in one or more of four nutrient-limited perpetual cultures, and the responses appeared to be correlated with relative nutrient availability (Table 8; Figure 6). Among the 14 steady-state populations, PHB was stored, and alkaline phosphatase and phosphate uptake activities increased whenever the ratio of C : P in the medium was at least 100 : 1. This ratio appears to elicit responses from *C. crescentus* appropriate to managing conditions of P-limitation.

Table 7 Chemotaxis[a]

Test solute[b]:	Hours	methionine				glucose				phosphate				NH_4Cl				
(10^x)M, x: Culture/mode		−2	−3	−4	−5	−2	−3	−4	−5	−2	−3	−4	−5	−1	−2	−3	−4	−5
N/I	27 h	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0
N/II	98 h	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0
	192 h	3.4	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0
	264 h	2.4	3.0	3.1	0	0	0	0	0	0	0	0	0	–	0	0	0	0
N/IV	361 h	1.6	0	1.9	2.2	0	0	0	0	0	0	0	0	–	4.4	1.8	0	0
	459 h	0	0	0	1.6	–	–	0	0	0	0	0	0	3.5	0	0	0	0
N/V	603 h	0	0	0	0	0	0	0	0	0	0	0	0	2.4	9.3	0	0	1.6
	650 h	2.6	0	0	0	0	0	0	0	0	2.0	0	0	9.9	2.7	1.9	1.7	1.7
	696 h	0	0	0	0	0	0	0	0	0	0	0	0	3.4	0	0	1.7	0
N/VI	795 h	0	0	0	0	0	0	0	0	0	0	0	0	4.9	1.8	0	0	0
	866 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C/II	188 h	–	–	1.3	1.5	–	–	–	–	–	–	0	0	–	–	–	–	–
	357 h	–	–	2.0	1.5	–	–	0	0	–	–	0	0	–	–	–	–	–
	429 h	–	1.4	0	2.4	–	–	0	–	–	–	0	–	–	–	–	–	–
	452 h	–	–	1.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	500 h	–	–	0	0	–	–	0	–	–	–	1.3	–	–	–	–	–	–
	645 h	–	–	1.7	0	–	–	0	–	–	–	0	–	–	–	1.8	–	–
C/III	789 h	–	–	0	0	–	–	0	–	–	0	0	0	–	–	–	–	–
	833 h	–	–	0	0	–	–	1.4	–	–	1.4	0	1.5	–	–	–	–	–
	908 h	–	–	0	0	–	–	0	–	–	–	0	0	–	–	–	–	–
	980 h	–	–	0	0	–	–	0	0	–	–	0	0	–	–	–	–	–

[a]Positive chemotaxis responses are indicated as the ratio of CFU μcap^{-1} containing test solution to CFU μcap^{-1} containing diluent. A response was regarded as positive when the ratio was at least twice the coefficient of variance observed among diluent-only samples—1.5 or greater for culture N, 1.3 or greater for culture C. Absence of response is indicated as zero; a dash (–) indicates that the test was not performed.
[b]Glutamate at 10^{-4} M was tested occasionally with culture C; the responses during C/II were 4.6 at 429 h, 2.1 at 645 h, and 0 at 500 h; and in C/III, 0 at all four test times.

Table 8 Responses to variation in relative nutrient fluxes

Culture/mode	Nutrient flux: μg h^{-1} per 10^9 CFU			Responses[a]							
	carbon	nitrogen	phosphorus	PHB	Pn	C-Up	N-Up	P-Up	APase	Stk	CT
J/I	8.1	3.8	0.106	0	+	–	0	0	0	0	–
J/II	10.5	3.6	0.091	++	0	–	0	+	+	+/–	–
J/III	13.0	3.7	0.085	++	0	–	0	+	+	+	–
J/IV	19.6	4.4	0.085	++	0	–	0	+	+	+	–
J/V	17.6	8.2	0.078	++	0	–	0	+	+	+	–
K/I	17.6	8.2	0.078	++	0	–	0	+	+	+	–
K/II	26.5	9.1	0.078	++	0	–	0	+	+	+	–
N/I[b]	13–47	1.2–4.7	0.12–0.47	++	+	0	+–0	+	–	–	0
N/II	15.4	1.0	0.15	+	+	0	+	0	–	–	+
N/III[b]	13–108	2–16	0.20–1.63	+	+	0	+	0	–	–	NM
N/IVa[c]	13.6	2.6	0.28	0	+	0	++	0	–	–	+
N/IVb[c]	10.5	2.0	0.22	0	+	+	+	0	–	–	+
N/V	16.6	3.2	0.086	++	0	0	0–+	+	–	–	0
N/VI	10.5	2.0	0.22	0	+	+	0	0	–	–	0
C/I	30.8	1.7	0.31	–	–	0	0/+	0	–	–	–
C/II	28.2	1.3	0.29	–	–	0	++	0	–	–	+
C/III	24.5	2.5	0.12	–	–	0	0	+	–	–	0

[a]Response to a particular nutrient limitation was regarded as positive by the following criteria. PHB storage at least 0.05 mg (+) or 0.10 mg (++) per mg dry weight. Polyphosphate storage at least 0.05 μg polyphosphate-P per mg dry weight. Substrate uptake, pmol min^{-1} mg^{-1} protein): phosphate at least 200, amino acid at least 1500, and glucose at least 100. Alkaline phosphatase was regarded as derepressed if at least 100 nmol of substrate was hydrolyzed min^{-1} mg^{-1} protein. Stalks were regarded as hypertrophied when three-banded stalks were twice the average length of non-stalked cells. Chemotaxis was interpreted as positive as indicated in Table 7.
[b]Steady state was not achieved.
[c]See Figure 4.

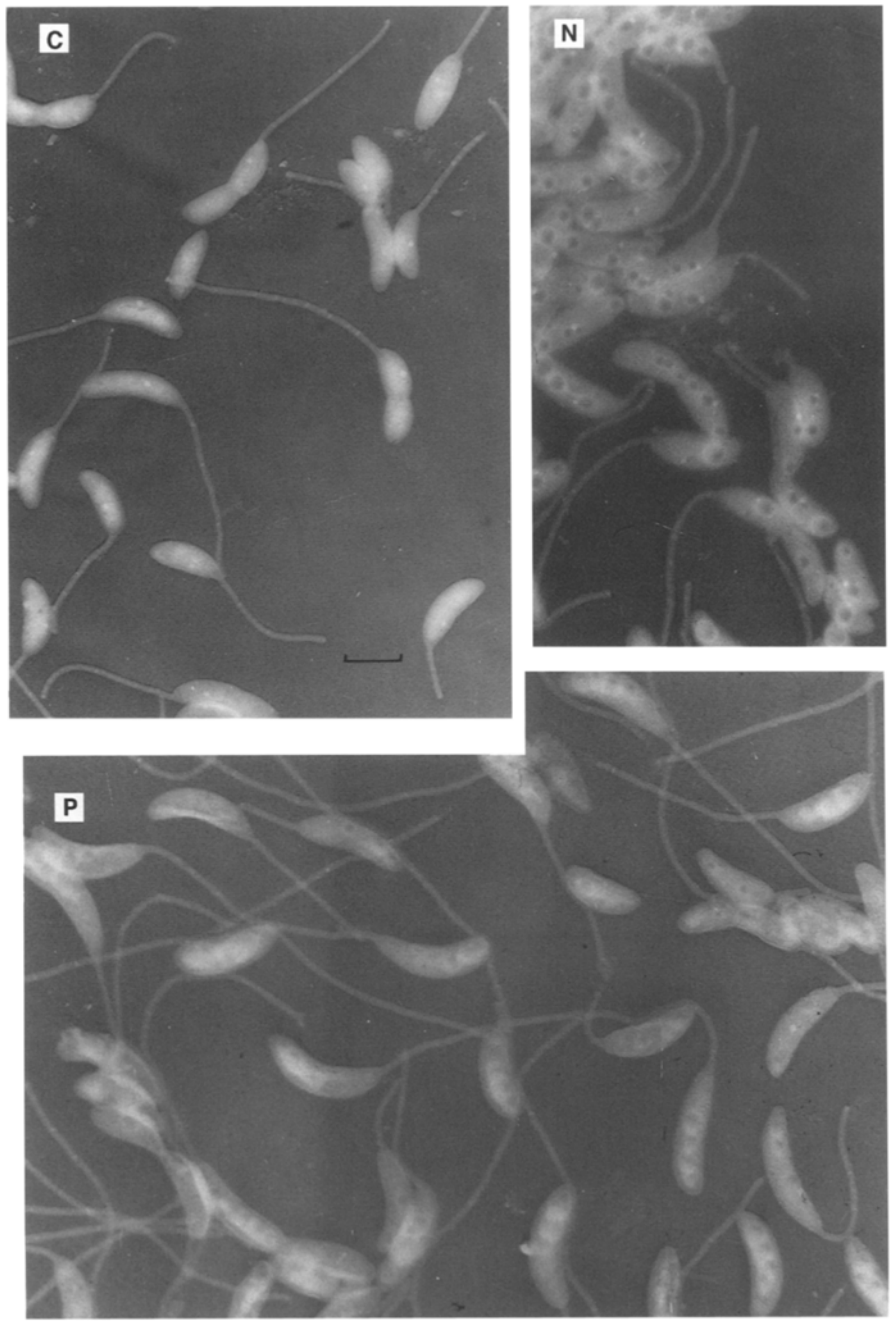

Figure 6 Appearance of *C. crescentus* cells in perpetual culture N. (C) Carbon-limited, mode VI (795 h). (N) Nitrogen-limited, mode II (264 h). (P) Phosphorus-limited, mode V (650 h). Shadowed electron micrographs, negative image; the marker is 1 μm and applies to all three micrographs.

These changes were accompanied by marked hypertrophy of the stalk, which further increased the capacity of the (stalked) cell to take up phosphate. Whenever the C : P ratio was not more than 100 : 1, polyphosphate was stored, further evidence that, in HiGg media, P available at greater than 1% (wt/wt) of the C available will serve *C. crescentus* as an excess nutrient.

A gravimetric C : N : P ratio of 48 : 9 : 1 successfully elicited two responses appropriate to C-limitation: enhanced rate of glucose uptake, and storage of polyphosphate. It did not elicit detectable chemotactic responses to gradients of glucose. Imposing manageable N-limitation was achieved only after the exquisite sensitivity of this organism to ammonium interference with ammonium assimilation was appreciated. Once achieved, however, by a C : N : P ratio of $>100 : <10 : >1$ in the medium, the cells responded by storing both PHB and polyphosphate, maintaining only relatively low levels of phosphate and glucose uptake activities while greatly increasing their capacity for the uptake of amino acids, and by exhibiting positive chemotactic responses to gradients of amino acids and ammonium chloride.

Discussion

In many studies employing chemostat-maintained perpetual cultures, nutrient flux to the culture is altered by changing the relative flow rate; however, that type of manipulation complicates interpretation of physiologic and other responses of the microorganisms because it superimposes changes in reproductive rate on changes in nutrient flux per cell. In the present studies, as in studies with a similar organism (strains of *Hyphomicrobium* [9,17]), changes in nutrient fluxes were achieved by changing substrate concentrations in the reservoir media while a constant relative flow rate and, therefore, a constant reproductive rate was maintained. This allowed the responses observed to be interpreted narrowly in terms of the relative flux of each of the varied nutrients. Among the four *C. crescentus* cultures, total reservoir carbon was varied 3.4-fold, nitrogen 4.3-fold, and phosphorus 9.3-fold. Population density varied 3.4-fold (as cells ml^{-1}) and 3.6-fold (as dry weight ml^{-1}). These narrow ranges nevertheless elicited responses over much wider ranges of rates of substrate uptake, and a seemingly all-or-none behavioral response.

Among the stable populations, each relative limitation—for carbon, for nitrogen, or for phosphorus—elicited an increase in the rate of uptake of a potential source of the limiting element. This could be achieved by cells in more than one conceivable way, but the most probable mechanism is synthesis of more of the relevant transport protein(s) and increase in the density of their distribution within the cell surface. This certainly seemed to be the case for cells in cultures J and K, where the rates of phosphate and of leucine uptake were significantly greater in stalked (S) than in non-stalked (N) cells when calculated per cell, and were still distinctly greater in S cells when calculated per unit protein. *C. crescentus* swarmers usually seem less active biosynthetically than stalked cells, do not replicate DNA, and are generally interpreted as cells specialized for dispersal [6,34,40,44]. However, uptake activities of S and N cells were not different when calculated per unit of cell surface area. The similarity of uptake rate per unit surface area in the two types of cells persisted as the rate of phosphate uptake increased, the rate of leucine uptake decreased, and cells of both types enlarged. Apparently, S cells grow faster because they eat faster.

The activity of only one enzyme was assayed during this study. As expected [60], the specific activity of alkaline phosphatase was greater in P-limited cells than in cells not strictly limited for P. However, the change was far smaller than the changes in rates of substrate uptake. This scavenging enzyme was not strongly repressible, a regulatory feature it shares with at least some catabolic enzymes [25] and assimilatory systems [13] in *C. crescentus*. Enzyme activity was not higher in S cells than in N cells (Table 3), even though it is a periplasmic enzyme [55], which implies that the intermembrane contents are not randomly distributed between the stalk and the cell body.

As expected, the morphology of *C. crescentus* cells (Table 4; Figure 6) was affected principally by the availability of inorganic phosphate, which retards stalk outgrowth [18,38,50] due to its interference with the supportive role of Ca^{2+} ions in stalk initiation and outgrowth [41,42]. In culture J, stalk length increased 2.8-fold at a constant absolute P flux as C and N fluxes increased. Decrease in absolute flux of P (from J/IV, to J/V and K/I) had little effect on stalk length, whereas further reduction in relative P flux by increased C and N fluxes (K/I to K/II) resulted in maximum stimulation of stalk outgrowth. As C and N were provided faster than P, the individual cells built more biomass per genome, yet surface : volume ratio increased in S cells as accelerated stalk elongation added surface in close proportion to increased cytoplasmic volume. In *C. crescentus*, a stalk band is added during each cell cycle [48,54], and acceleration of stalk outgrowth was reflected in cultures J and K in a progressive increase in average interband distance from 0.7 μm $band^{-1}$ in J/I through 1.9 μm $band^{-1}$ in K/II. Shown for three-banded stalks only (Table 4), interband distance was constant within each population among stalks with up to 9–10 bands.

C. crescentus possesses genes and gene products similar in composition to chemotaxis genes and proteins in other organisms. Mutations in the genes have been mapped [2,14,53], and the relevant proteins have been shown to be selectively inherited and active in the swarmer sibling [3,33; reviewed in 6,52]. Nevertheless, chemotaxis that causes non-random distribution of *C. crescentus* swarmers in a liquid environment has not proved detectable. Chemotaxis and its loss as a consequence of mutation has been inferred on the basis of whether cells were able to grow out into soft agar, to reverse direction while swimming under a cover slip, or to methylate and demethylate appropriate membrane proteins [2,14,53]. Wild-type *C. crescentus* is positive for all of these traits; mutant clones unable to form wide colonies in soft agar often lack one or another of the other traits and are interpreted as chemotaxis mutants [2,14,53]. Our own attempts to employ an Adler-type microcapillary assay with *C. crescentus* swarmers collected during or at the end of exponential growth in HiGg or in peptone/yeast extract media were not successful.

Two explanations for the seeming lack of distributively significant chemotaxis were considered. One was the possibility that in the oligotrophic environments where caulobacters typically occur, gradients of soluble, assimilable nutrients may be so shallow and/or short-lived that they neither stimulate nor reward chemotactic responses, and so *C. crescentus*' chemosensory genes and proteins serve solely for aerotaxis. Alternatively, these oligotrophic organisms might spare the biosynthetic effort of maintaining a complete chemosensory apparatus unless overall development is limited by the need for a specific nutrient; in response to a specific limitation, the apparatus might be provided with a specific receptor for a solute other than O_2 (which they need in all environments), possibly a component of a transport system. This would improve the potential of the organism to balance its diet by locating a source of the missing nutrient. A similar hypothesis was examined by Terraciano and Canale-Parola with *Spirochaeta aurantia* grown in sugar-limited chemostat cultures [58]. Chemotactic responsiveness to glucose (or to xylose) was distinctly enhanced when glucose (or xylose) was the limiting nutrient, and enhancement was sugar-specific. Each enhanced response was maximal at a 'peak D'; however, because reservoir concentrations of the two sugars were different, each maximum

enhancement actually occurred at about the same sugar flux, *viz*, at the flux that delivered >3.2 and <4.0 μg sugar-C h^{-1}.

In the present studies with *C. crescentus*, only N-limitation appeared to stimulate chemotactic responsiveness sufficient for detectability in a microcapillary assay. However, in contrast to results with *S. aurantia*, responsiveness in this case was acquired behavior, not an enhancement of constitutive responsiveness. In the cell cycle of *C. crescentus* growing in a N-limited environment, responsiveness of the swarmer to a N gradient might lead it to a site of greater N availability, where it could develop into a stalked cell able to manage limitations of other nutrients, such as C or P. In a N-adequate environment, random motility and/or probably-constitutive aerotaxis would serve to separate the swarmer from its non-motile sibling and thereby reduce competition between them for the scarce resources to which they are accustomed.

Extreme N-limitation was unintentionally imposed on *C. crescentus* during two attempts to impose C-limitation by increasing the flux of NH_4Cl (and, in N/III, also of phosphate) without a proportional increase in glutamate flux. The adverse effects on the culture were unanticipated from preliminary studies with batch cultures, and not readily interpreted with the help of batch cultures prepared in the reservoir media during the course of the experiment. Further, *C. crescentus* strain 2NY66R is not a glutamate auxotroph; it grows in batch cultures in glucose/NH_4Cl medium. It can also grow in batch cultures with glutamate as sole N-source, but at a lower growth rate (5.8 h/doubling) than when NH_3 is the sole N-source, and with glutamate as sole source of both C and N, but more slowly still (nearly 12 h/doubling). With glucose available and either N-source alone, significant PHB is stored (up to 30% of dry wt), yet total yield per input C is half that achieved in batch cultures with the same total C and N, but with both N sources present (Poindexter, unpublished).

The explanation offered here for the unsuitability of reservoir media I and III for perpetual culture N has two components. The first lies in the intermittency of individual cell growth in chemostat cultures. Regardless of the mechanics of delivery (dropwise, point-stream, diffusion of a gas into solution, solubilization within the growth vessel, etc), nutrients are consumed by the population almost as rapidly as they become available. Newly available nutrients run a gauntlet of substrate-avid cells and do not diffuse throughout the population before they are consumed; this is the meaning of nutrient limitation. When mixing is adequate, each cell receives its share (is fed) as frequently as each other cell, but not every cell will be fed out of every delivery. Thus, while the feeding rate per cell is constant, feeding is not a constant activity of each cell. Between meals, various changes may occur within the cells. Such changes are traditionally attributed to D (= f/V), but the concept of intermittent feeding of each cell makes many recorded 'effects of D' (maintenance energy, derepression of enzymes, the results of competition between strains or species), as well as mechanisms of simultaneous limitations, much easier to comprehend as familiar physiologic consequences of interrupted cellular growth. In the present instance, 'effects of D' were not available as an explanation because D was not varied.

A second component of the explanation arises from *C. crescentus*' metabolism. This organism lacks glutamic dehydrogenase and is dependent on an ammonium-repressed glutamine synthetase : glutamine/oxoglutarate aminotransferase (GS/GOGAT) system for ammonium assimilation [13]. The media provided for modes I and III of culture N contained less glutamate-N than ammonium-N, and less than 10 μg C per μg N; with a limiting supply of C as glucose, the population probably consumed some of the small supply of glutamate as a source of C and energy. In the chemostat culture, as soon as the glutamate arriving in each drop of medium was consumed, the cells were in an environment approximating the suspending medium used to impose 'ammonia shock' and thereby increase the inactivation of glutamine synthetase by adenylylation [4]. The population starved for nitrogen because the supply of nitrogen in the form of ammonia was excessive relative to the supply of C, and especially to the supply of glutamate. The similarity of the aberrant cells in culture N to cells grown in batch cultures with high initial phosphate concentrations, with ethylene glycol bis-(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA), or without added $CaCl_2$ [42], suggested that morphogenesis was impaired by the high flux of phosphate relative to assimilable N. C-limitation reduced availability of glutamate, making the cells more susceptible to ammonium shock and less able to assimilate N, and consequently more susceptible to retardation of morphogenesis and reproduction by phosphate. And so the population collapsed.

Morphogenesis in *Hyphomicrobium* is phosphate-sensitive and appears Ca-dependent, as in *C. crescentus* [41], and a similar problem was encountered in chemostat cultures of *Hyphomicrobium* ZV620 in a medium containing more than 10 mM phosphate [17]. Strain ZV620, like *Hyphomicrobium* X [5], can synthesize NADP-dependent glutamic dehydrogenase, but not when grown N-limited at a C : N ratio greater than 8. Nutrient-limited ZV620 tolerated up to 50 mg ammonium-N per mg dry weight of cells at C : N = 6, but at C : N above about 9, the maximum tolerated flux appeared to be 24 mg ammonium-N h^{-1} per mg dry wt. At higher NH_4^+ fluxes, 'the cells were sensitive to residual methanol, and wash-out, wall growth and the formation of small, misshapen cells was observed.'

Whether the shocking ammonia flux or the relatively high phosphate flux to *C. crescentus* and *Hyphomicrobium* cells impaired in ammonium assimilation was the immediate cause of morphogenetic and reproductive deterioration, the implication is the same: under nutrient-limited conditions, these organisms appear to be intolerant of high fluxes of inorganic nutrients, just as they are of organic substances. Our recognizing this may improve their cultivability from samples of natural populations, their industrial applicability, and our general understanding of the nature of oligoheterotrophy in microorganisms.

Acknowledgements

The authors are grateful to Barry Rosen for challenging us to measure the surface area of curved, tapered, lumpy bac-

terial cells, and to Richard Novick for providing the planimeter required. We are also grateful to Sylvia Johnson for her perseverance through months of negative microcapillary assays, and for her genuine sense of loss when the long-awaited responsiveness was abolished by shifting the culture to phosphorus limitation.

This work was supported by grant number AI-15467 from the National Institutes of Health and by grant number PCM-7903659 from the National Science Foundation. Early portions of these studies were conducted at The Public Health Research Institute of The City of New York, Inc.

References

1 Adler J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J Gen Microbiol 74: 77–91.
2 Alley MRK, SL Gomes, W Alexander and L Shapiro. 1991. Genetic analysis of a temporally transcribed chemotaxis gene cluster in *Caulobacter crescentus*. Genetics 129: 333–342.
3 Alley MRK, JR Maddock and L Shapiro. 1992. Polar localization of a bacterial chemoreceptor. Genes Dev 6: 825–836.
4 Bender RA, KA Jensen, AD Resnick, M Blumenberg, F Foor and B Magasanik. 1977. Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. J Bacteriol 129: 1001–1009.
5 Brooke AG, MG Duchars and MM Attwood. 1987. Nitrogen assimilation in the facultative methylotroph *Hyphomicrobium* X. FEMS Microbiol Lett 41: 41–45.
6 Brun YV, G Marcynski and L Shapiro. 1994. The expression of asymmetry during caulobacter cell differentiation. Annu Rev Biochem 63: 419–450.
7 Cohen-Bazire G, WR Sistrom and RY Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J Cell Comp Physiol 49: 25–68.
8 Daugherty DD and SF Karel. 1994. Degradation of 2,4-dichlorophenoxyacetic acid by *Pseudomonas cepacia* DB01(pRO101) in a dual-substrate chemostat. Appl Environ Microbiol 60: 3261–3267.
9 Duchars MG and MM Attwood. 1989. The influence of C:N ratio in the growth medium on the cellular composition and regulation of enzyme activity in *Hyphomicrobium* X. J Gen Microbiol 135: 787–793.
10 Duetz WA, C deJong, PA Williams, JG van Andel. 1994. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl Environ Microbiol 60: 2858–2863.
11 Egli T, V Lendenmann and M Snozzi. 1993. Kinetics of microbial growth with mixtures of carbon sources. Antonie van Leeuwenhoek 63: 289–298.
12 Egli T and JR Quayle. 1986. influence of the carbon:nitrogen ratio of the growth medium on the cellular composition and the ability of the methylotrophic yeast *Hansenula polymorpha* to utilize mixed carbon sources. J Gen Microbiol 132: 1779–1788.
13 Ely B, ABC Amarasinghe and RA Bender. 1978. Ammonia assimilation and glutamate formation in *Caulobacter crescentus*. J Bacteriol 133: 225–230.
14 Ely B, CJ Gerardot, DL Fleming, SL Gomes, P Frederikse and L Shapiro. 1986. General nonchemotactic mutants of *Caulobacter crescentus*. Genetics 114: 717–730.
15 Gottschal JC. 1986. Mixed substrate utilization by mixed cultures. In: Bacteria in Nature (Poindexter JA and ER Leadbetter, eds), vol 2, pp 261–292, Plenum Press, New York.
16 Gottschal JC. 1993. Growth kinetics and competition—some contemporary comments. Antonie van Leeuwenhoek 63: 299–313.
17 Grazer-Lampart SD, T Egli and G Hamer. 1987. Growth of *Hyphomicrobium* ZV620 in the chemostat: regulation of NH_4^+-assimilating enzymes and cellular composition. J Gen Microbiol 132: 3337–3347.
18 Haars EG and JM Schmidt. 1974. Stalk formation and its inhibition in *Caulobacter crescentus*. J Bacteriol 120: 1409–1416.
19 Harder W and L Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Phil Trans R Soc Lond [Biol] 197: 459–480.
20 Harder W and L Dijkhuizen. 1983. Physiological responses to nutrient limitation. Annu Rev Microbiol 27: 1–23.
21 Harder W, JG Kuenen and A Matin. 1977. Microbial selection in continuous culture. J Appl Bacteriol 43: 1–24.
22 Jannasch HW. 1969. Estimation of bacterial growth rates in natural waters. J Bacteriol 99: 156–160.
23 Jannasch HW and T Egli. 1993. Microbial growth kinetics: a historical perspective. Antonie van Leeuwenhoek 63: 213–224.
24 Koch AL. 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. Adv Microb Physiol 6: 147–217.
25 Kurn N, I Contreras and L Shapiro. 1978. Galactose catabolism in *Caulobacter crescentus*. J Bacteriol 135: 517–520.
26 Landa AS, EM Sipkema, J Weijma, AACM Beenackers, J Dolfing and DB Janssen. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl Environ Microbiol 60: 3368–3374.
27 Larson RJ and JL Pate. 1976. Glucose transport in isolated prosthecae of *Asticcacaulis biprosthecum*. J Bacteriol 126: 282–293.
28 Lauffenburger D, R Aris and K Keller. 1982. Effects of cell motility and chemotaxis on microbial population growth. Biophys J 40: 209–219.
29 Lowry RH, NJ Rosebrough, AL Farr and RJ Randall. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.
30 Malthus T. 1830. A summary view of the principle of population. In: An Essay on the Principle of Population, pp 219–272, Penguin Books, Harmondsworth, UK.
31 Matin A and H Veldkamp. 1978. Physiological basis of the selective advantage of a *Spirillum* sp in a carbon-limited environment. J Gen Microbiol 105: 187–197.
32 Meers JL, DW Tempest and CM Brown. 1970. 'Glutamine(amide):2-oxoglutarate amino transferase oxido-reductase (NADP)', an enzyme involved in the synthesis of glutamate by some bacteria. J Gen Microbiol 64: 187–194.
33 Nathan P, SL Gomes, K Hahnenberger, A Newton and L Shapiro. 1986. Differential localization of membrane receptor chemotaxis proteins in the *Caulobacter* predivisional cell. J Mol Biol 191: 433–440.
34 Newton A. 1984. Temporal and spatial control of the *Caulobacter* cell cycle. In: The Microbial Cell Cycle (Nurse P and E Streiblova, eds), pp 51–75, CRC Press, Cleveland.
35 Onishi T, RS Gall and ML Mayer. 1975. An improved assay of inorganic phosphate in the presence of extralabile phosphate compounds: application to the ATPase assay in the presence of phosphocreatine. Anal Biochem 69: 261–267.
36 Poindexter JS. 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol Rev 28: 231–295.
37 Poindexter JS. 1978. Selection for nonbuoyant morphological mutants of *Caulobacter crescentus*. J Bacteriol 135: 1141–1145.
38 Poindexter JS. 1979. Morphological adaptations to low nutrient concentrations. In: Strategies of Microbial Life in Extreme Environments (Shilo M, ed), pp 341–356, Verlag Chemie, Weinhelm.
39 Poindexter JS. 1981. Oligotrophy: fast and famine existence. Adv Microb Ecol 5: 63–89.
40 Poindexter JS. 1981. The caulobacters: ubiquitous, unusual bacteria. Microbiol Rev 45: 123–179.
41 Poindexter JS. 1984. Role of prostheca development in oligotrophic aquatic bacteria. In: Current Perspectives in Microbial Ecology (Klug MJ and CA Reddy, eds), pp 33–40, American Society of Microbiology.
42 Poindexter JS. 1984. The role of calcium in stalk development and in phosphate acquisition in *Caulobacter crescentus*. Arch Microbiol 138: 140–152.
43 Poindexter JS. 1987. Bacterial responses to nutrient limitation. Symp Soc Gen Microbiol 41: 283–317.
44 Poindexter JS. 1992. Dimorphic prosthecate bacteria: the genera *Caulobacter, Asticcacaulis, Hyphomicrobium, Pedomicrobium, Hyphomonas*, and *Thiodendron*. In: The Prokaryotes (Balows A, HG Truper, M Dworkin, W Harder and KH Schlieffer, eds), 2nd edn, pp 2176–2196, Springer-Verlag, New York.
45 Poindexter JS and LF Eley. 1983. Combined procedure for assays of poly-β-hydroxybutyric acid and inorganic polyphosphate. J Microbiol Meth 1: 1–17.
46 Poindexter JS and JG Hagenzieker. 1981. Constriction and septation during cell division in caulobacters. Can J Microbiol 27: 704–719.
47 Poindexter JS and JG Hagenzieker. 1982. Novel peptidoglycans in *Caulobacter* and *Asticcacaulis* spp. J Bacteriol 150: 332–347.

48 Poindexter JS and JT Staley. 1996. *Caulobacter* and *Asticcacaulis* stalk bands as indicators of stalk age. J Bacteriol 178: 3939–3948.
49 Porter JS and JL Pate. 1975. Prosthecae of *Asticcacaulis biprosthecum*: system for the study of membrane transport. J Bacteriol 122: 976–986.
50 Schmidt JM and RY Stanier. 1966. The development of cellular stalks in bacteria. J Cell Biol 28: 423–436.
51 Schneider WC. 1957. Determination of nucleic acids in tissues by pentose analysis. In: Methods in Enzymology (Colowick SP and NO Kaplan, eds), vol III, pp 680–684, Academic Press, New York.
52 Shapiro L. 1985. Cell differentiation in *Caulobacter*. TIG 1: 317–321.
53 Shaw P, SL Gomes, K Sweeney, B Ely and L Shapiro. 1983. Methylation involved in chemotaxis is regulated during *Caulobacter* differentiation. Proc Natl Acad Sci USA 80: 5261–5265.
54 Staley JT and TL Jordan. 1973. Crossbands of *Caulobacter crescentus* stalks serve as indicators of cell age. Nature (Lond) 246: 155–156.
55 Steinman HM and B Ely. 1990. Copper zinc superoxide dismutase of *Caulobacter crescentus*: cloning, sequencing, and mapping of the gene and periplasmic location of the enzyme. J Bacteriol 172: 2901–2910.
56 Stove JL and RT Stanier. 1962. Cellular differentiation in stalked bacteria. Nature (Lond) 196: 1189–1192.
57 Tempest DW and OM Neijssel. 1981. Metabolic compromises involved in the growth of microorganisms in nutrient-limited (chemostat) environments. In: Trends in the Biology of Fermentations for Fuels and Chemicals (Hollaender A, ed), pp 335–356, Plenum Publishing, New York.
58 Terraciano JS and E Canale-Parola. 1984. Enhancement of chemotaxis in *Spirochaeta aurantia* grown under conditions of nutrient limitation. J Bacteriol 159: 173–178.
59 Tommassen J and B Lugtenberg. 1980. Outer membrane protein e of *Escherichia coli* K-12 is co-regulated with alkaline phosphatase. J Bacteriol 143: 151–157.
60 Torriani A. 1960. Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. Biochim Biophys Acta 38: 460–469.
61 Trevelyan WE and JS Harrison. 1952. Studies on yeast metabolism. 1. Fractionation and microdetermination of cell carbohydrates. Biochem J 50: 298–303.