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Adaptive responses of *Ralstonia eutropha* to feast and famine conditions analysed by flow cytometry

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Abstract

Results obtained by flow cytometry allow conclusions to be drawn about how the physiological states of *Ralstonia eutropha* JMP134 are connected with survival strategies under distinct growth conditions. During both feast and famine conditions the cells were found to proceed through sharply separated phases of life. Two sources of carbon and energy, one poor (0.02% phenol) and one rich (0.2% pyruvate and 0.1% yeast extract) were chosen to study the cellular responses. Despite the major differences in carbon source, when growth stages of the bacteria on the two substrates were characterised in batch growth, only minor differences were found in the time course of the membrane potential related fluorescence intensity (MPRFI). This also applied to the rRNA content and the size-correlated forward scatter (FSC) signal of the cells, both of which increased to high levels during the (early) exponential growth phase. On the rich medium, DNA synthesis initially occurred in an uncoupled manner, then a high rate of PHB formation followed when nutrients began to be limiting. Under famine conditions, the cellular responses were much more complex. PHB was synthesised, then DNA synthesis occurred in a 'eukaryotic' mode, to be succeeded by renewed PHB synthesis. To obtain defined cell physiological states, the chemostat technique was used in addition to batch experiments. The results obtained clearly indicated that key events in cell physiology, including initiation of DNA replication and overflow metabolism, occurred in a hierarchically ordered manner and were tightly correlated with changes in the environmental conditions of the bacterial cells.

Keywords: Bacterial population dynamics; Cell cycle; Life strategies; Flow cytometry

1. Introduction

Microbes have evolved complex, hierarchically ordered strategies enabling them to withstand both feast and famine conditions. Principal elements of these strategies are mechanisms that help safeguard the genetic information and maintain an energy budget by replicating DNA and storing energy as and when appropriate.

Growth and multiplication represent the forward strategy of survival of populations. Thus, the growth rate of a strain, which is determined

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by the specific DNA replication time, is a very important parameter when competitive situations arise. In cases of imbalanced substrate supplies and temporary changes in nutrient sources, overflow metabolism is induced, which is thought to be part of a survival strategy (Babel, 1992; Babel et al., 1993). An example is the synthesis of poly-β-hydroxybutyrate (PHB). Accumulation of this compound increases the survivability of an individual, since it is an energy source that can be made available at a later stage, thereby allowing continuation of the bacterial life cycle (Ackermann et al., 1995). Much attention was drawn concerning the investigation of physiological states of *Escheria coli* on the single cell level under changing life situations (Skarstad et al., 1986; Cooper, 1991; Bernander et al., 1995). However, cell states are described for other bacterial strains, too, as shown in studies on Azotobacter vinelandii (Allman et al., 1992), *Micrococcus lutus* (Kaprelyants and Kell, 1993), *Salmonella typhimurium* and *Alteromonas haloplanktis* (Lebaron and Joux, 1994) as well on marine populations (Monger and Landry, 1993; Button et al., 1996), greatly enhancing the ability to understand and follow growth, death and multiplication activity.

In this study, the gram-negative bacterium *Ralstonia eutropha* JMP134 was used as a model organism for elucidating bacterial life cycle strategies under conditions of both feast and famine nutrient availability. The strain is capable of producing PHB, it is ubiquitously found in the environment, and it is very often used to metabolise and detoxify xenobiotics (formerly described as *Alcaligenes eutrophus*, Busse and Auling, 1992).

Measurement of parameters connected with these key life processes in cell populations as a whole will give the usual mean values, but will not necessarily help to understand their implications at the level of individual cells, or the distribution of different states within the population. Therefore, in this study, flow cytometry was used, since it provides single-cell data on these processes, as a highly localised method.

We have followed, in detail, the dynamics of the synthesis and content of DNA, a major cellular component, responsible in large part for the existence of the individual. PHB was monitored to identify points in the bacterial life cycle where strategies must be initiated to survive critical situations. Changes in forward light scatter (FSC), which is strongly correlated to cell size, membrane potential related fluorescence intensity (MPRFI), a measure of the energetic status of the cells, and rRNA content, indicating the growth rate potential, were chosen as analytical parameters for identifying the physiological state and the overall fitness of individual cells.

We attempted to clarify whether relationships between the above five physiological features at the level of the single cell are advantageous to get information about the adaptive responses of *R. eutropha* JMP134 in changing environmental conditions.

2. Materials and methods

2.1. Strain and cultivation

R. eutropha JMP134 was kindly supplied by H.-J. Knackmuss, University of Stuttgart. The strain was grown aerobically at 30°C, at pH 7.0, in 150 ml standard medium (containing 310 mg phosphate/l) with equimolar concentrations of KH_2PO_4 and K_2HPO_4, 0.76 g l^{-1} NH_4Cl and the following salts (with their respective concentrations listed in mg l^{-1}): $MgSO_4.7H_2O$ (71.2), $ZnSO_4.7H_2O$ (0.44), $MnSO_4.H_2O$ (0.61), $CuSO_4.5H_2O$ (0.78), $NaMoO_4.2H_2O$ (0.25), $Fe(SO_4)_2.7H_2O$ (4.98). The carbon and energy sources used were phenol (0.02%) and pyruvate (0.2%) + yeast extract (0.1%). The cells were periodically pre-cultivated on either 0.02% phenol or on 0.2% pyruvate + 0.1% yeast extract within 12-h intervals, prior to analyses, by inoculation into fresh batches of standard medium. This was done to avoid lag-phases and to provide similar test conditions with regard to growth on the different substrates.

Chemostat cultivations were carried out in transient state systems in an Infors ISF 100 laboratory bioreactor (Einsbach, Germany) with 1 l working volume at a growth temperature of 30°C. Further equipment used is fully described by Müller and Babel (1996). Using this system, any deviations

from stable hydrodynamic conditions could be observed and the mass flow balances could be adjusted if necessary. The pH was held constant at 6.8 by titration with 0.8 M NaOH or 0.8 M HCl as required. The dissolved oxygen concentration in the fermentation broth ranged from 50 to 80% of air saturation as determined by an Ingold oxygen electrode. For the transient state course, *R. eutropha* JMP134 was pre-cultivated on 0.2% pyruvate, and was grown continuously with a dilution rate of 0.05 h^{-1} for a day. Following this, the carbon and energy source was shifted to 0.01% phenol, and the cells were grown with the same dilution rate, 0.05 h^{-1}, for a further 24 h. The dilution rate and other cultivation conditions, such as pulse additions of nutrients, could then be selected as desired. Using the transient state method, the bacterial cells were grown on phenol in increasing nutrient flow rates (R. Müller et al., 1995).

The key kinetic parameters of growth (maximum growth rate [μ_{max}] and generation time [τ]) on the two different substrates were estimated both from batch and continuous cultivation experiments. Biomass was measured off-line via optical density (OD: 700 nm, $d_{cuvette} = 10$ mm). Cells were harvested at various growth phases by sedimentation at 5000 rev./min for 5 min, usually at room temperature, for assessment of the membrane potential at 4°C. Before DNA, PHB and rRNA staining, the cells were incubated in a NaN_3-solution (10%). The cells should be preserved in such a way for at least 10 min, but not more than 2 months. For estimating the membrane potential, the cells were stained immediately after sampling and measured by flow cytometry.

2.2. Preparation and staining of the cells

2.2.1. Estimation of the DNA content

In this study, the multiplication of each sample was determined by measuring the fluorescence intensity after staining the cells with the dye 4′,6-diamidino-2′-phenylindole (DAPI). The preserved cells were centrifuged for 5 min at 5000 rev./min, washed in NaCl–phosphate buffer (0.4 M Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.2) and resuspended at a concentration of 3×10^8 cells ml^{-1}. The cell concentration of the samples was determined using the Coulter Counter technique. These cells were then centrifuged a second time and were stained using a modification of a standard procedure according to Otto (1994). They were treated with 1 ml solution A (2.1 g citric acid/0.5 g Tween 20 in 100 ml bi-distilled water) for 10 min. Optimal staining conditions were determined by testing both different staining times and dye concentrations (Fig. 1). The following process was selected. After treating the cells with solution A, they were washed and resuspended in 2 ml solution B (0.24 μM DAPI, 400 mM Na_2HPO_4, pH 7.0) for 25–120 min at room temperature before measuring their fluorescence intensity.

2.2.2. Estimation of the PHB content

Flow cytometry is an established method for detecting intracellular accumulation of PHB in many bacterial strains (Srienc et al., 1984; Ackermann et al., 1995; Fouchet et al., 1995). In this study, the PHB content was measured using the dye Nile Red (S. Müller et al., 1995), as follows: 40 μl of a Nile Red stock solution (1 mg ml^{-1} acetone), added to 3×10^8 cells ml^{-1}, was needed to reach optimal alignment in 10 min. The equilibrium between the free and the intracellularly bound Nile Red is disturbed by dye crystallisation caused by its hydrophobic nature. For that reason the time interval allowed for reaching the staining equilibrium must be kept constant.

2.2.3. Estimation of the membrane potential

Cell state and cell cycle dependent variations of bacterial membrane potential have been shown in the case of *Escherichia coli* (Monfort and Baleux, 1996) and *Acinetobacter calcoaceticus* 69-V (Müller et al., 1996). In order to assess the membrane potential related fluorescence intensity (MPRFI), the cells were resuspended in 20 mM imidazole buffer (pH 7.0), and immediately adjusted to 3×10^8 cells ml^{-1}. The composition of the staining solution was taken from Shapiro (1988): 100 μg $diOC_6(3)$ dissolved in 166 μl dimethylformamide and topped up with ethanol to 3.25 ml. For *R. eutropha* JMP134, optimal alignment was defined by testing different dye

concentrations and staining times and calculation of the CV-values (coefficient of variation, i.e. standard deviation divided by mean value) from the derived histograms (Fig. 2). Accordingly, 5 μl of the dye stock solution was added to 3×10^8 cells ml^{-1} for 10 min. Non-specific staining of intracellular compounds could be microscopically observed after just 15 min incubation with the dye. All measurements were carried out at 4°C.

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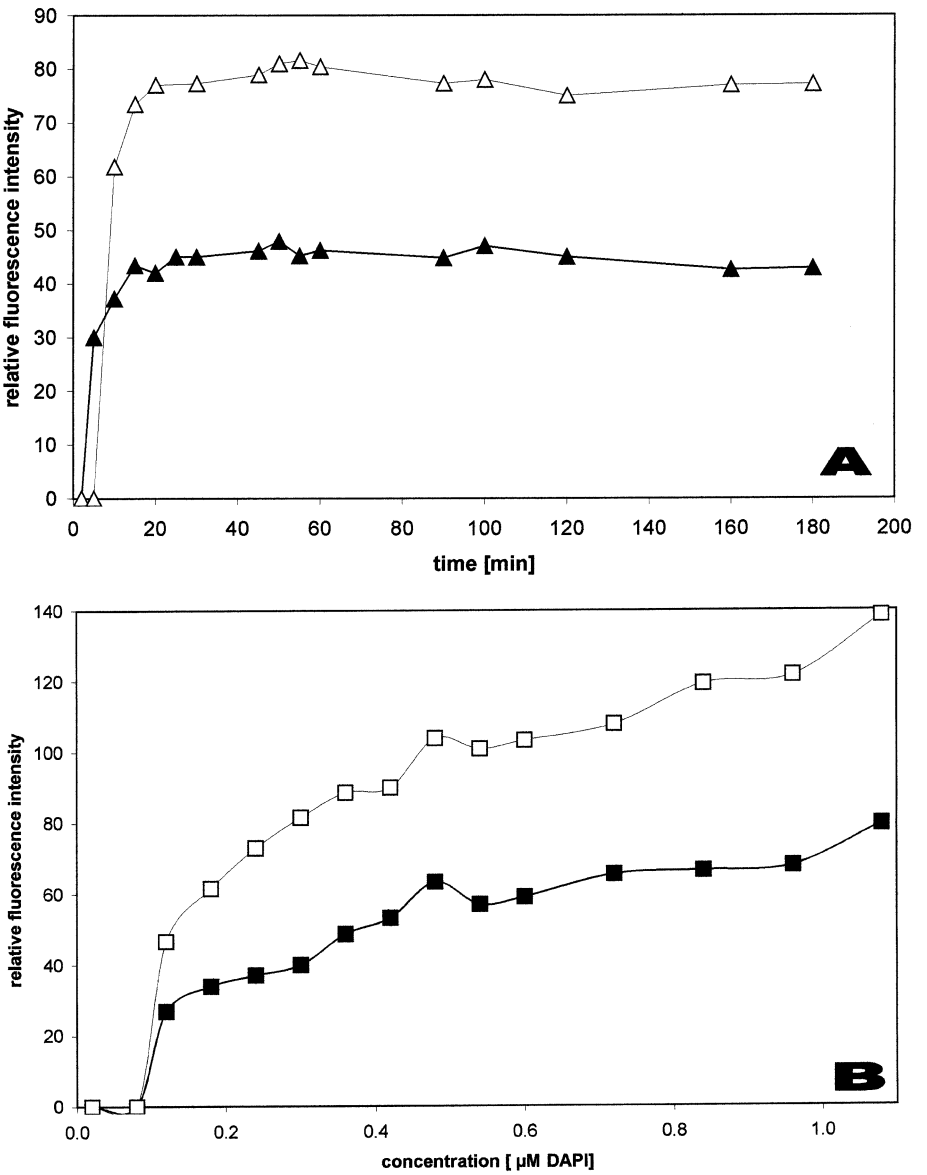

Fig. 1. Variation of DNA staining with respect to the staining time (A) and dye concentration (B). Effects of the staining time are shown for a DAPI concentration of 0.24 μM, and effects of the dye concentration are shown for a staining time of 30 min. The cells were grown on 0.2% pyruvate + 0.1% yeast extract and harvested during the mid-exponential growth phase. The maximum DNA fluorescence intensity of each sub-population, containing one and two chromosomes was calculated.

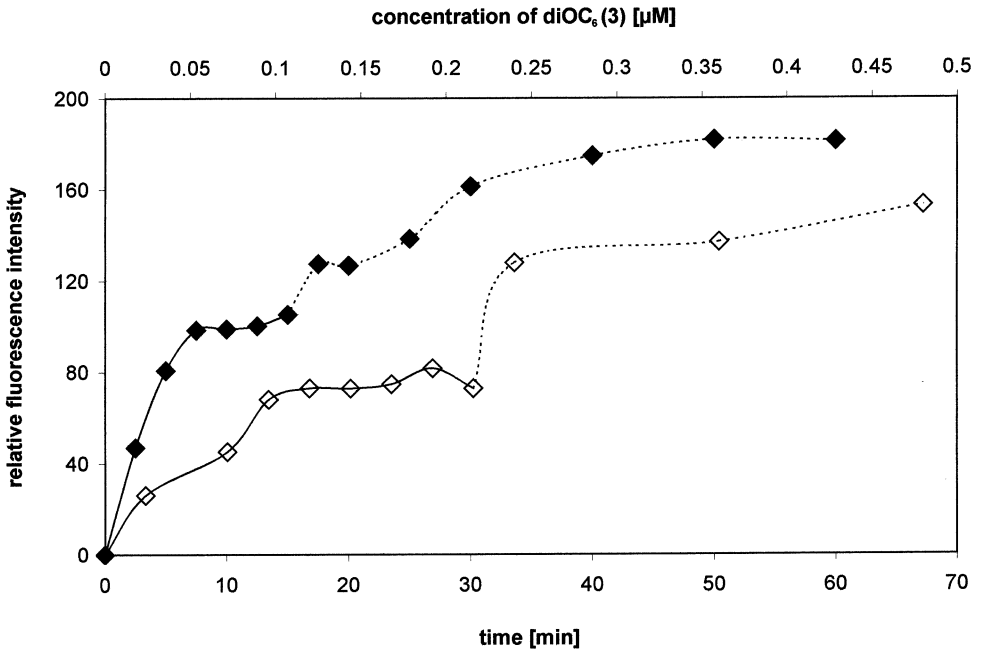

Fig. 2. Variation of MPRFI staining with respect to the staining time (◆) and dye concentration (◇). Effects of the staining time are shown for a diOC$_6$(3) concentration of 0.12 µM, and effects of the dye concentration are shown for a staining time of 10 min. The cells were grown on 0.2% pyruvate + 0.1% yeast extract and harvested during the early exponential growth phase.

cell-bound dye to changes in the membrane potential was verified by analysing the effects of the ionophores valinomycin and gramicidin. Cells grown on 0.2% pyruvate + 0.1% yeast extract were harvested during the exponential growth phase, counted, and treated with different concentrations of the two ionophores during the staining procedure and measured via flow cytometry. Low concentrations of gramicidin (5 µM, Fig. 3A) decreased the MPRFI, whereas low concentrations of valinomycin (5 µM, Fig. 3B) were shown to cause hyperpolarisation. Higher concentrations of both ionophores (500 µM) were found to dramatically reduce the MPRFI.

During cultivation of *R. eutropha* JMP134, the level of the MPRFI is influenced by variations in cell surface area. To distinguish between (and evaluate) effects of cell surface area and increased membrane potential on the MPRFI, the ratios of the MPRFI K_{max} values (estimated by the maximal channel number of the relative fluorescence intensity) vs. the K_{max} values of the FSC signals were calculated.

2.2.4. Estimation of the RNA content

Group- and strain-specific oligonucleotide probes were directed against the 16S rRNA and 23S rRNA of *R. eutropha* JMP134 for estimating the rRNA content. Primarily, in-situ hybridisation is used to determine the taxonomic membership of a bacterial strain (Amann et al., 1995); however, in this investigation it was found to be a reliable method for measuring rapid changes in the physiological activity of *R. eutropha* JMP134. The procedure adopted was performed according to the methods of Wallner et al. (1993) and Herrmann et al. (1997), using cells preserved in 10% NaN_3; 2–4 µl (about 10^6 bacterial cells) were hybridised in 50 µl hybridisation buffer (pH 7.4, 0.9 M NaCl, 10 mM Tris/HCl, 0.1% sodium dodecyl sulphate) and 1.5 ng μl^{-1} fluorescence-marked oligonucleotide probes (BETA: L-Sc-bProt-1227-a-A-17 and/or AEU: S-Ss-Alc-0647-a-A-18) for 2 h at 48°C. Following this, the cells were washed in hybridisation buffer at 48°C for 15 min and resuspended in 1 ml NaCl–phosphate buffer (130 mM NaCl, 10 mM $Na_2HPO_4/Na_2HPO_4/NaH_2PO_4$,

pH 8.4). The fluorescent dyes CY3 and Cy3.5 (Amersham) are bound at the 5′-end of the oligonucleotide probes, respectively (monolabelled, MWG-Biotech).

The rRNA content was detected by an off-line method, which is able to detect quick changes in the cellular rRNA content of the bacterial cells. However, both this technique and MPRFI measurement have limited applicability. Although both methods were most sensitive to exponentially growing cells, there are situations where these methods would not be valid. Membrane potential measurements are not suitable, in contrast to rRNA staining, if the cells multiply at low dilution rates. They show non-specific binding of the dye under such circumstances. Stationary phase cells could definitely not be reliably evaluated by these means, for the same reason. Measurement of the rRNA content is quite often inappropriate during the late stationary growth phase, however,

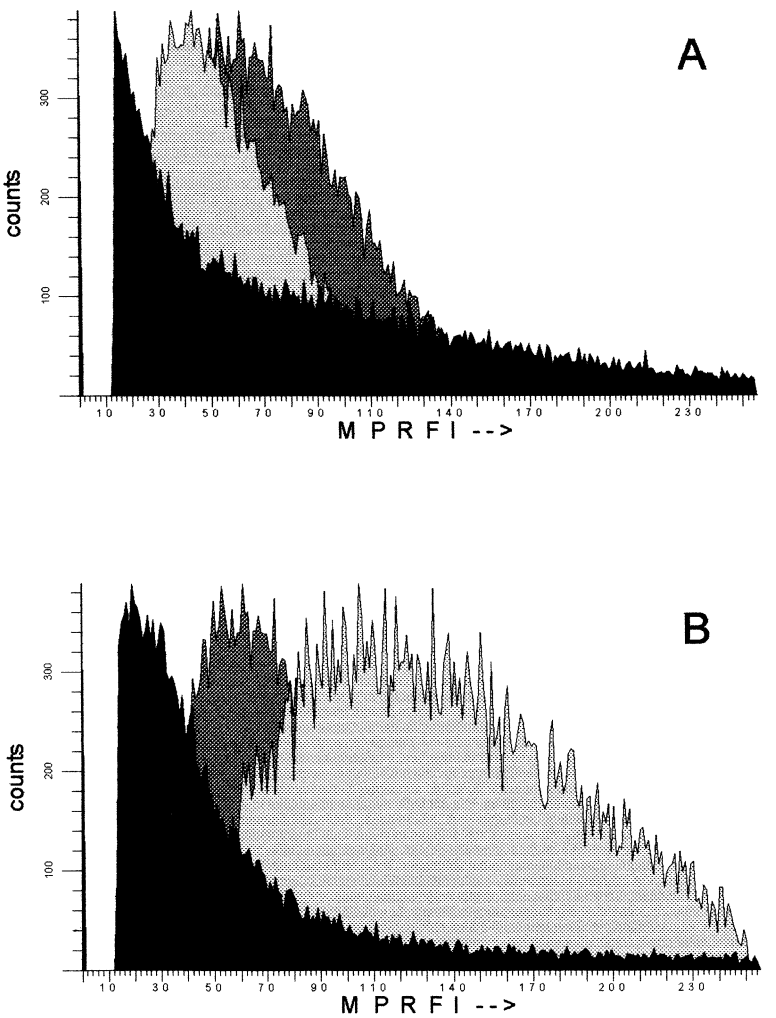

Fig. 3. The effects of different concentrations of the ionophores gramicidin (A) and valinomycin (B) on MPRFI were investigated. *R. eutropha* JMP134 was grown on 0.2% pyruvate + 0.1% yeast extract and harvested during the exponential growth phase. A: control (grey), 5 μM gramicidin (pale), 500 μM gramicidin (dark); B: control (grey), 5 μM valinomycin (pale), 500 mM valinomycin (dark).

due to impermeability of the membranes to the rRNA probes, and the low amounts of cellular rRNA found at this cultivation stage.

2.3. Flow cytometry

A modular flow cytometer was used with a stream-in-air flow chamber and an argon-ion-laser as light source (Innova 304, Coherent, USA). The measuring parameters were forward light scatter (3.5–9.6°) and fluorescence emission at 90° to the illuminating beam (DAPI: $\lambda_{exc} = 333.6–363.8$ nm, $\lambda_{em} = 400–580$ nm, power output: 370–400 mW; $diOC_6(3)$: $\lambda_{exc} = 488$ nm, $\lambda_{em} = 500–550$ nm, power output: 650 mW; CY3: $\lambda_{exc} = 514$ nm, $\lambda_{em} = 540–620$ nm, power output: 1000 mW; Cy3.5: $\lambda_{exc} = 528$ nm, $\lambda_{em} = 580–630$ nm, power output: 500 mW). Light scatter analysis was applied principally for examination of the cell size. The photomultiplier gains were linear, and a total of 32 000 cells were analysed in each sample at a rate of 250–350 cells per s. Optimum alignment was based on the optimised signals of 1.0 μm (BB, ref. 441286 and NYO, ref. 9003536, Polyscience Inc., respectively) and 0.75 μm (YG, ref. 442191, Polyscience Inc.) diameter fluorescent beads. Data were visualised using WinList 2.01.

2.4. Microscopy

Samples were routinely viewed using phase-contrast and fluorescence microscopy (BH-2, Olympus). The cells were excited by ultraviolet light from a 100 W mercury arc lamp. Blue fluorescence was viewed through a UG1 filter (band pass 360). Red and green fluorescence were observed using BP545 and BP495 filters, respectively.

2.5. Analyses

Phenol was determined by High Pressure Liquid Chromatography (HPLC) using the method of Oh and Tuovinen (1990). The concentration of pyruvate was measured using a DX-100 ion chromatograph with an Ion Pac ASA4-SC Analytical (4×250 mm) ion exchange column running on a suppressor basis. The elution medium was 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ (1:1). It was necessary to substitute the NH_4Cl in the medium with $(NH_4)_2SO_4$ in the case of growth on pyruvate, because high concentrations of chloride ions interfere with the detection of this substrate.

3. Results

3.1. Growth on rich substrate

Pyruvate (0.2%) + 0.1% yeast extract were used as carbon and energy source to establish feast growth conditions. At the beginning of batch-cultivation *R. eutropha* JMP 134 presented a single chromosome equivalent (Fig. 4). During the first hour of cultivation, the cells increased in size, and a minor proportion of the cells began to synthesise PHB (7%). In the next 2 h most of the cells synthesised DNA in an uncoupled manner, indicating that more than one chromosome was synthesised during the strain-specific replication time. Within this brief time interval, these cells did not accumulate PHB and the generation time (τ) was estimated to be about 1.28 h (Table 1). In the fourth hour of cultivation, the nutrients began to be growth limiting (Fig. 5A). No uncoupled DNA synthesis was measurable after this stage of cultivation, and the generation time increased to more than 2 h (2.56 h). Subsequently, the cells multiplied at a lower rate in a 'eukaryotic' manner, i.e. we observed two sharply separated phases of replication and cell division. It must be noted that the cell density (OD) did not correlate strongly with these findings. The cells showed a dramatic increase in absorption (Fig. 5A), but this was caused by increases in the refractive index of the cells. The limited growth conditions clearly induced the cells to start overflow metabolism. The maximum PHB content was found at the fourth hour of cultivation and slowed down during the next 3 h. After 8 h of cultivation, no PHB content could be detected (Fig. 5B). The cells also underwent cell division, leading to a uniform population of small cells, each with a single chromosome equivalent.

MPRFI changed in correlation to the different growth phases of *R. eutropha* JMP134 (Fig. 5C). During the lag, and the following early, and mid-

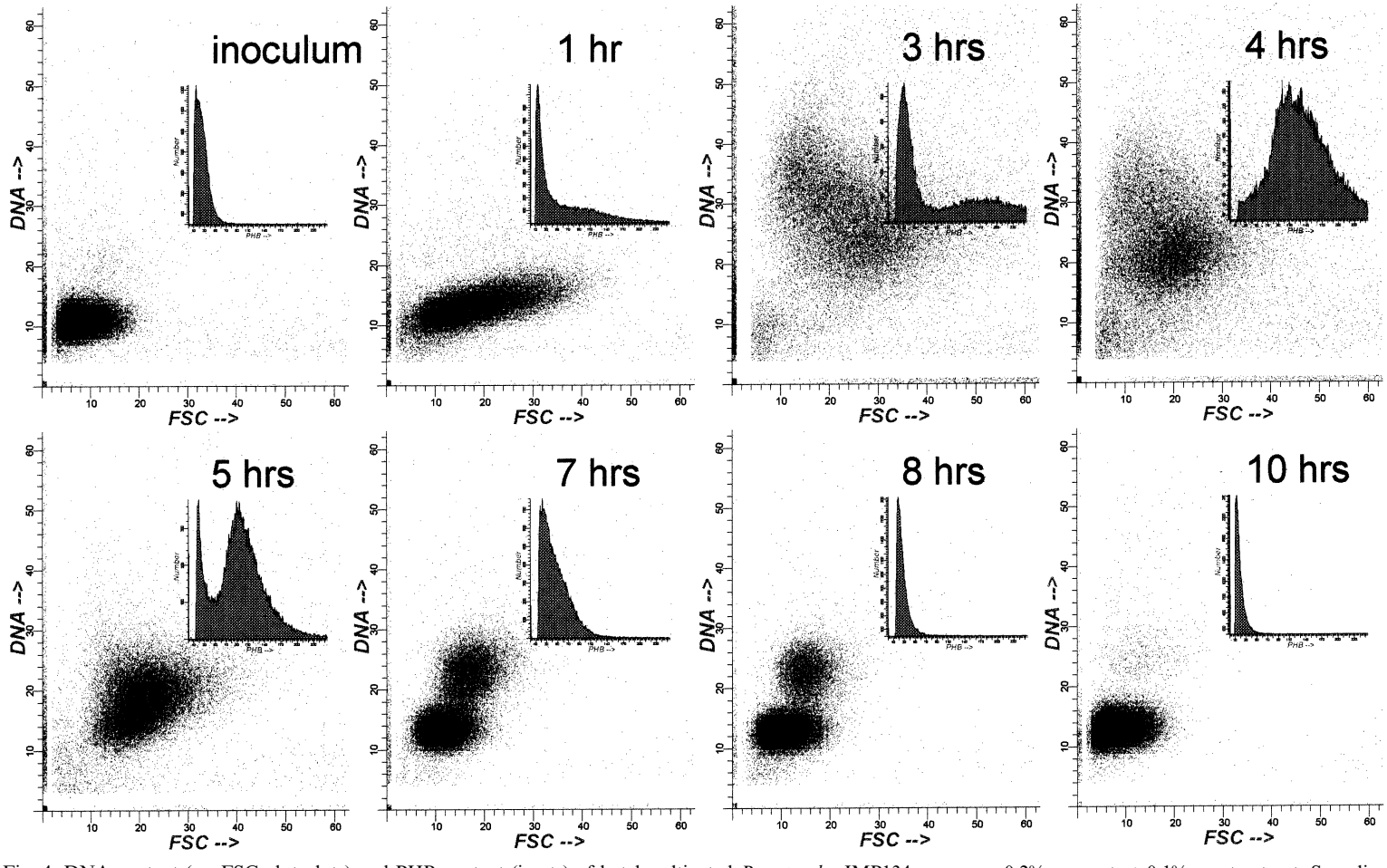

Fig. 4. DNA content (vs. FSC, dot plots) and PHB content (insets) of batch-cultivated *R. eutropha* JMP134 grown on 0.2% pyruvate + 0.1% yeast extract. Sampling times are indicated. The lower dots in the DNA plots represent cells with a single chromosome (e.g. in the case of the inoculum); the other dots represent cells with two or more chromosomes.

Table 1
Growth rates and generation times of *R. eutropha* JMP134 during growth on the two substrates: 0.2% pyruvate + 0.1% yeast extract, and 0.02% phenol[a]

Substrate	Cultivation time (h)	Growth rate (h^{-1})	Generation time (h)	MPRFI (maximum relative values)	% C2	rRNA (maximum relative values)	Rate of increase (MPRFI vs. FSC)
0.2% pyruvate +	0–1	n.d.	n.d.	30.1	24.33	17.1	1.51
0.1% yeast	1–4	0.54	1.28	85.9	89.05	57.8	0.53
extract	4–8	0.27	2.56	76.1	67.62	24.1	0.17
0.02% phenol	0–1	n.d.	n.d.	37.2	2.98	20.1	−3.12
	1–4	0.31	2.23	131.9	73.92	130.3	1.29
	4–9	0.19	3.64	104.8	57.22	85.5	0.16

[a] The growth rate μ (h^{-1}) and the generation time τ (h) were estimated from typical growth courses by measuring the optical density (OD). The proportion of replicating cells (% C2) was calculated by gating the two-dimensional plotted DNA-histograms (vs. FSC). The rRNA-content and the MPRFI were presented as maximum values of the relative fluorescence intensity at specific times of cultivation. The specific staining of the membrane potential was validated by estimating the rate of increase in the MPRFI vs. FSC ratio.

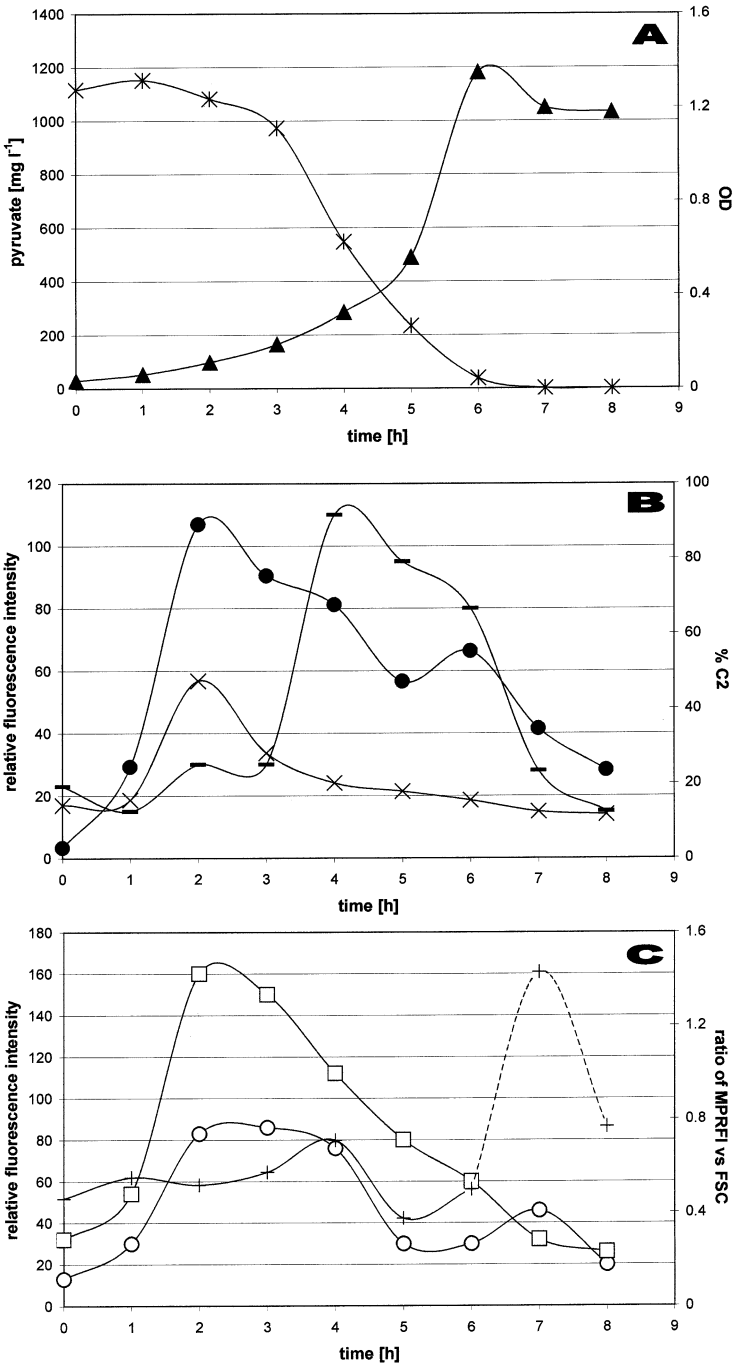

Fig. 5. Growth characteristics of batch-cultivated *R. eutropha* JMP134 on 0.2% pyruvate + 0.1% yeast extract. Diagram A shows optical density (OD, [▲]) and consumption of pyruvate [∗]. Diagram B shows both the maximal channel numbers of the rRNA [×] histogram values and PHB content [–]. The multiplication activity was calculated by gating cells having two or more chromosomes [●]. Diagram C shows the maximal channel number of each histogram for MPRFI [○], the FSC signal [□], and the ratio of these values (MPRFI vs. FSC) [+]. The broken line represents samples stained non-specifically.

exponential growth phases, the MPRFI/FSC ratios remained constant, or even slightly increased, implying an authentic increase in membrane potential. This was proved by the positive values derived for $\Delta ratio_{MPRFI/FSC}/\Delta t$ (Table 1). During limitation of the carbon source (between the 4th and 6th hours), the MPRFI rapidly declined to a much lower intensity. Thus, when the pyruvate was exhausted, MPRFI was depressed. At the 7th hour of batch-cultivation a small increase in MPRFI was registered, related (even at this early stage) to non-specific staining of the cells, as observed microscopically.

As for MPRFI, both the cellular rRNA content and the FSC signal increased strongly during the first 2 h of batch cultivation. After the onset of limited growth, between the 3rd and the 4th hour, the cellular rRNA content decreased quickly, and the FSC signal had a more subdued intensity (Fig. 5B,C). The PHB content of the cells had only a minor influence on the measured FSC signal because of the scatter angle used (3.6–9.6°).

3.2. Growth on poor substrates

Phenol (0.02%) was used as carbon and energy source to establish famine growth conditions. Initially, compared to growth on 0.2% pyruvate + 0.1% yeast extract, a somewhat higher proportion of cells produced PHB (22%) during the first 3 h of cultivation (Fig. 6). This was observed before DNA synthesis occurred. The cells increased in size within this time interval and subsequently started multiplying ($\mu = 0.31\ h^{-1}$, $\tau = 2.23$ h, Table 1) and the cellular PHB content decreased to zero (in the third to fifth hour of cultivation). The small red fluorescence distributions of the insets (at the 4th and 5th hour) were due to non-specific staining of the neutral lipids of the cellular membranes, and could not be related to the substance PHB. In contrast to growth on the rich substrate, described above, no uncoupled DNA synthesis was observed under the famine conditions.

After 4 h of cultivation, two sharply separated sub-populations (with cells having one or two chromosomes) were detectable, indicating sustained cell division. The multiplication rate slowed down (between the 4th and the 7th hours) and the generation time was found to have increased ($\tau =$ 3.64, Table 1). Simultaneously with the onset of substrate limitation (in the 6–7th hours, Fig. 7A), PHB was overflow-produced, providing an indication of restricted growth conditions. At the 8th hour, maximal PHB contents were found (Fig. 7B). The levels decreased quickly thereafter, due to cell division and/or PHB utilisation as an energy reserve, leading to a unique population with only one chromosome per cell and no PHB.

During the first 2 h of cultivation there was a strong correlation between the increases in the FSC signal and the MPRFI, as shown by the decreasing $\Delta ratio_{MPRFI/FSC}/\Delta t$ values (Table 1). Following this, the MPRFI increased to a high level and positive values were recorded for $\Delta ratio_{MPRFI/FSC}/\Delta t$ (Fig. 7C). Contemporaneously with the decreasing multiplication activity which followed this phase (Fig. 7B, 4–7th hour), the MPRFI was lower in intensity, but stable. The calculated MPRFI/FSC ratio appeared to be balanced in the mid-exponential growth phase, but it increased after the 7th hour: an effect associated with non-specific intracellular lipid staining.

The size-related FSC signal of the cells, and the rRNA content, peaked during the second and third hours of cultivation. During the mid-exponential growth phase, up to the early stationary phase, the rRNA content remained stable at a low level. A homogeneous population appeared, consisting of small cells, having only one chromosome each, a low PHB content and only minor amounts of rRNA.

3.3. Transient state cultivation on phenol

R. eutropha JMP134 cells were grown continuously with increasing dilution rates of 0.05–0.276 h^{-1} on phenol, using the transient state cultivation method. This was done to investigate cellular characteristics under constant growth conditions, using the above parameters. A typical course of cultivation induced by increasing nutrient flow rates is shown in Fig. 8. The given dilution rates corresponded to the growth rates. The DNA content is shown as the portion of cells (%) with two chromosomes. The increase in the abundance of

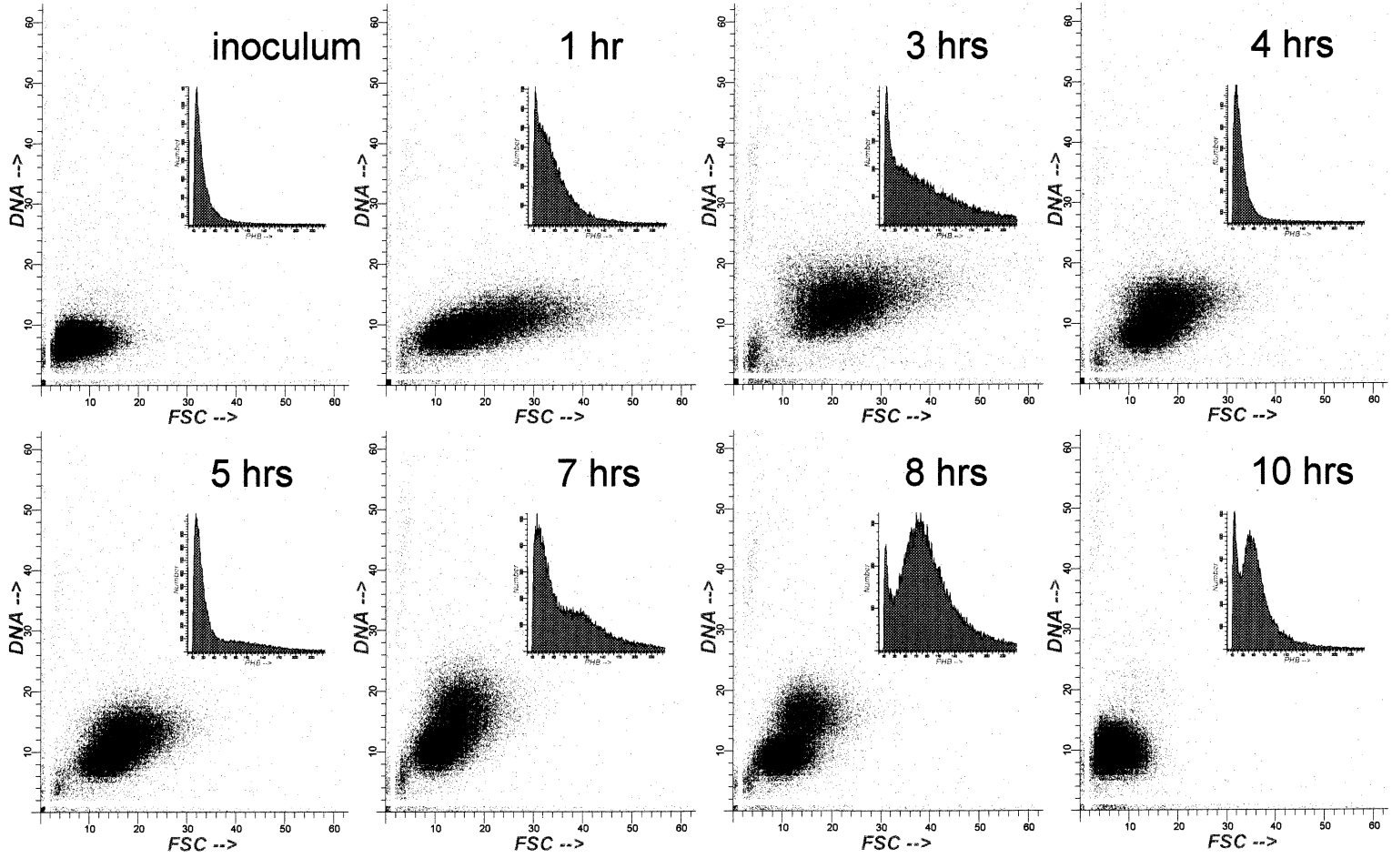

Fig. 6. DNA content (vs. FSC, dot plots) and PHB content (insets) of batch cultivated *R. eutropha* JMP134 cells grown on 0.02% phenol. Sampling times are indicated. The lower dots in the dot plots represent cells with a single chromosome content (e.g. in the case of the inoculum); the other dots, cells with two chromosomes.

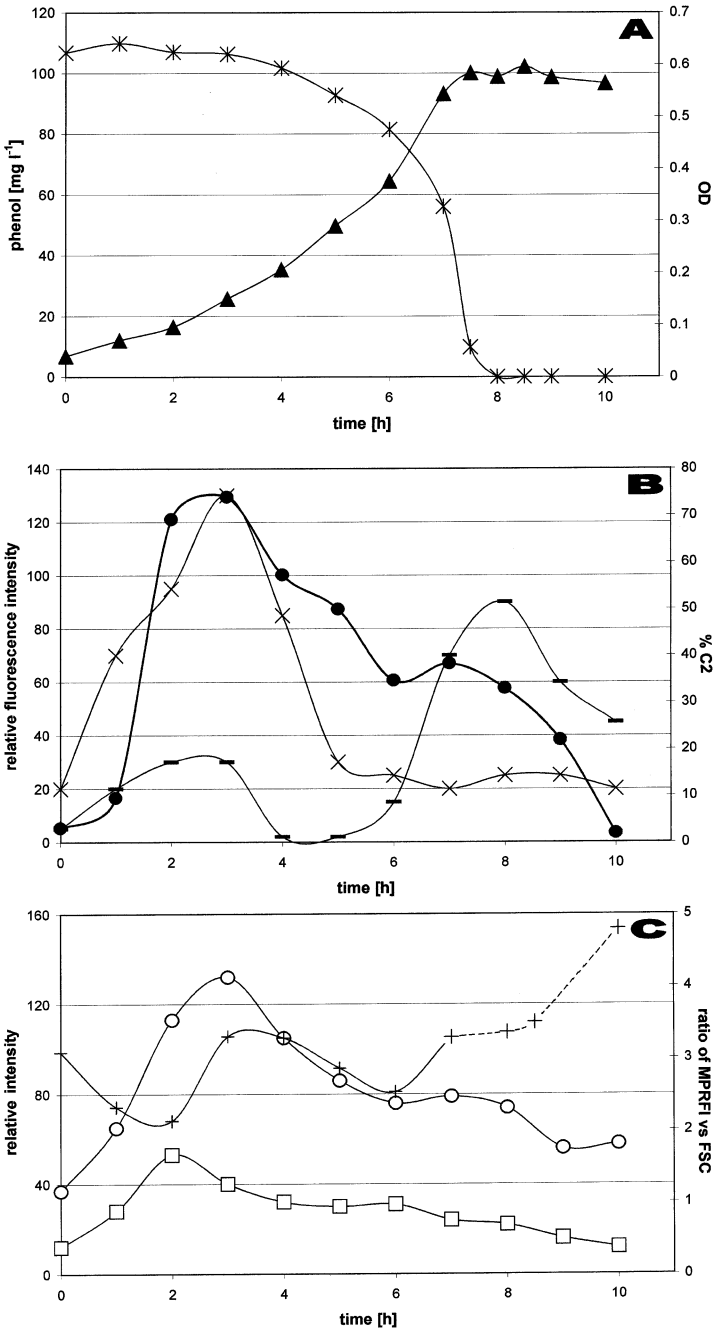

Fig. 7. Growth characteristics of *R. eutropha* JMP134 cells, batch cultivated on 0.02% phenol. Diagram A shows optical density (OD, [▲]) and the consumption of phenol [*]. Diagram B shows the maximal channel numbers of the rRNA [×] histogram values and PHB content [–]. The proliferation activity is calculated by counting cells with two chromosomes [●]. Diagram C shows the maximal channel number of each histogram for MPRFI [○] and the FSC signal [□], and the ratio of these values (MPRFI vs. FSC) [+]. The broken line represents samples stained non-specifically.

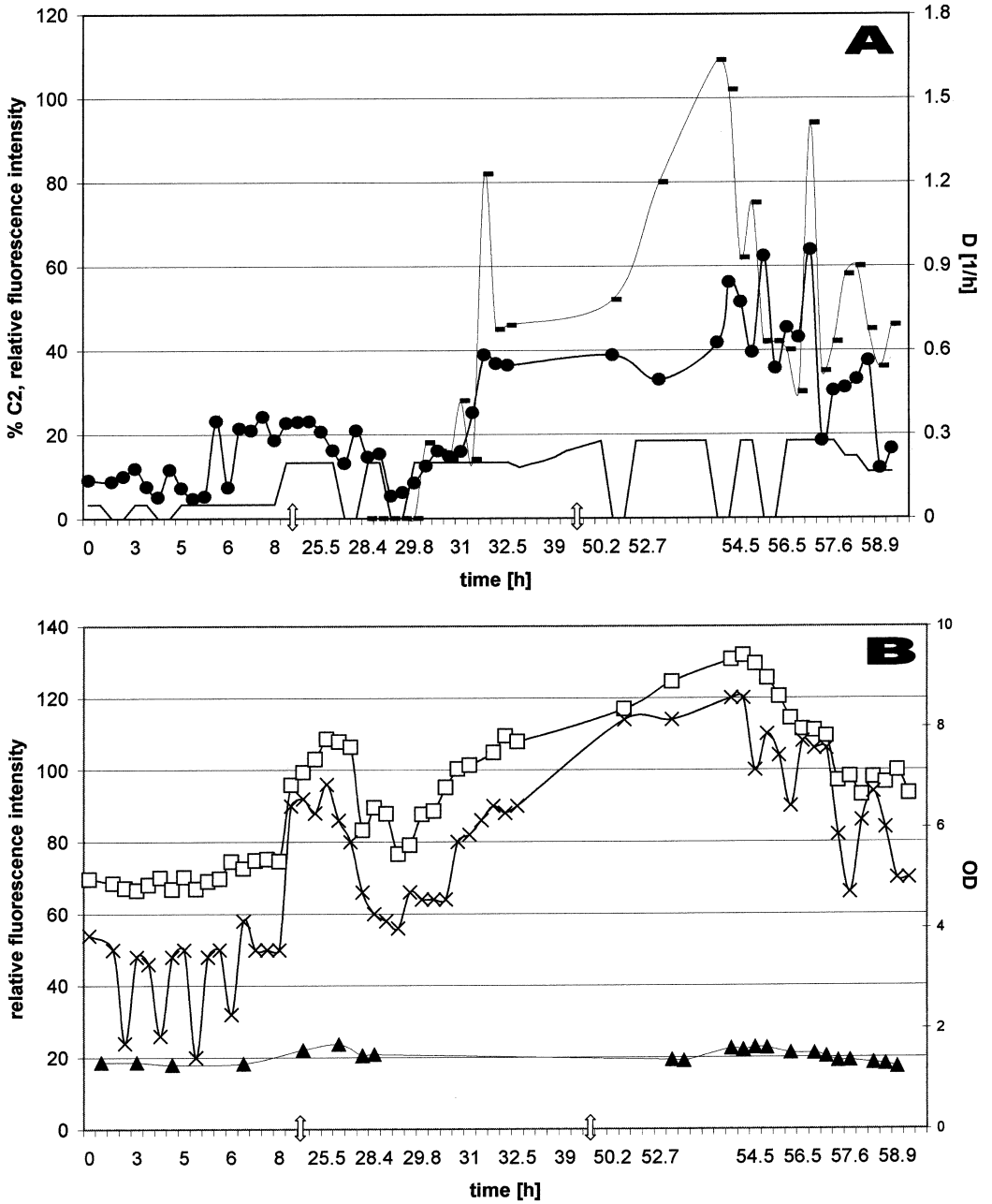

Fig. 8. Transient state cultivation of *R. eutropha* JMP134 on phenol at increasing dilution rates ($\Delta D = 0.025$, $\Delta t = 2$ h in undisturbed growth, except near the carbon limitation points). Multiplication (diagram A) was estimated from the proportion of cells (%) having two chromosomes (*). The amount of PHB (diagram A) is shown in relative values according to the maximal channel number of each histogram (–). The optical density was measured off-line (▲, B), the rRNA content (× , B) and the cell size related forward light scatter (□, B) were measured by estimating the maximal channel number of each histogram.

these multiplying cells correlated very well with the increase in the dilution rate (Fig. 8A). As observed in batch cultivation on 0.02% phenol, no uncoupled DNA synthesis occurred.

Substrate depletion was introduced to disturb the stable growth conditions of the process and to investigate the cellular responses to nutrient depletion. This prompted a rapid reduction in the C2 population, suggesting that carbon deficiency induced immediate cell division, under both low and high dilution rates (Fig. 8A). After adding fresh substrate, DNA replication was initiated anew.

At low dilution rates no PHB was synthesised. However, at a dilution rate of 0.2 h^{-1} and after substrate depletion, PHB production started. In contrast to the behaviour shown during batch cultivation, the cells were apparently able to synthesise PHB concurrently with DNA. However, this was observed only when carbon depletion was provoked several times, and the dilution rate was equal to, or higher than, 0.2 h^{-1} (after the 30th hour). Then, repeatedly, a large and rapid increase in the cellular PHB content was observed shortly after the substrate depletion and the following phenol pulse. However, this PHB accumulation diminished abruptly with further carbon limitation. This behaviour of *R. eutropha* JMP134 is found very precisely between the 50th and 57th hours of cultivation. After manipulating the culture in such a way, the cells tended to multiply and undertake overflow metabolism at the same time. This was observable for dilution rates between 0.22 and 0.275 h^{-1}. The diminished PHB content appeared to be induced by cell division, because the proportion of multiplying cells decreased simultaneously with the decline in PHB. The rRNA content also increased with the dilution rate, and oscillated in a similar manner to the DNA content in response to carbon depletion (Fig. 8B). Measuring the FSC signals and, even more so, the OD values, gave little information about the cellular states during the shown cultivation course.

4. Discussion

Adaptive responses of *R. eutropha* JMP134 towards feast and famine growth conditions were analysed flow cytometrically by measuring the dynamics of DNA, rRNA, and PHB contents, the strength of the MPRFI and the FSC signal at the single cell level. It was found that the organism successfully responds to contrasting life situations in various ways, involving both multiplying and storing strategies. Multiplication is favoured in balanced conditions, whereas *R. eutropha* JMP134 responds to unbalanced conditions with overflow metabolism, especially the accumulation of PHB.

There is a general survival strategy, characterised by maintenance and multiplication of the genetic information, which has the highest priority. We have shown that this strategy is governed by the quality of the substrates, which induces quantitative differences. In growth on rich substrate (0.2% pyruvate + 0.1% yeast extract), uncoupled DNA synthesis was found. The growth characteristics on the poor substrate tested (0.02% phenol) is typical of a slowly growing bacterium and proceeds in a 'eukaryotic' way. This interpretation is widely accepted in literature concerning *E. coli*. We may assume that this phenomenon reflects the relative affinities of *R. eutropha* JMP134 to the different substrates.

How much and how often the DNA is multiplied provides an indication of substrate quality and availability (as well as the influence of the genetic disposition of the strain assessed). The faintest traces of substrates were required to perform replication (not considering the time which is needed for initiation of the cell cycle), which could occur even on poor substrates at low concentrations, as was shown in the continuous experiments. The substrate is, above all, used to carry out the cell cycle.

In none of the situations described did overflow metabolism, essentially a mechanism of energy storage and survival, occur simultaneously with multiplication activity under balanced growth conditions. This was true both during batch cultivation (for a short time) and for undisturbed continuous growth. However, if there was any stress, e.g. imbalance, which prevented undisturbed replication, then *R. eutropha* JMP134 turned to overflow metabolism, producing PHB. If substrate depletion was artificially induced, and followed by a substrate pulse, overflow metabolism clearly occurred simultaneously with multiplication. After a very short

period, first PHB was synthesised and then DNA. Unexpectedly, under disturbed continuous cultivation conditions, PHB was quickly synthesised and degraded (within 1 or 2 h). The synthesised PHB was diminished by cell division and appeared to be used either for continuing DNA replication and completing cell division, or for maintaining turnover processes and cell-homeostasis which is clearly seen for both substrates.

If we assume that the membrane potential is an expression of the extent to which cells are able to generate energy from substrates they have obtained and, consequently, a measure of the energetic state of the cells, the highest membrane potential is anticipated at the early exponential growth phase of the batch processes, and this was observed both for rich and poor substrate supply. As the rRNA content and the growth rate are proportional to the available substrate we were not surprised that MPRFI and the rRNA content of the cells showed the same time course.

The synthesis of PHB was obviously not directly related to the existence of a high membrane potential. Additionally, we have observed that the method of MPRFI measurement began to lose validity when growth conditions became imbalanced. During the periods of PHB production and decreasing DNA synthesis, and especially, in the case of growth on phenol at low dilution rates, the MPRFI method was insufficiently reliable, due to non-specific staining of intracellular lipids. At this point of cultivation, the cells were certainly not dead, but their membrane potential was not strong enough to bind the dye used directly to the inner membrane bilayer. Using $diOC_6(3)$ we intended to find the most active moment of cellular activity, which we believe would be characterised by high membrane potential and strong proliferative activity.

When the external substrates are exhausted and the intracellular energy source is no longer available, the cells tend to become smaller, and adopt a spherical form, finally becoming coccoidal spheres with low surface/volume ratios. This was observed independently of the carbon source offered. This behaviour is symptomatic of the onset of vegetative dormancy, and is typical for oligotrophic bacteria.

In summary, under conditions of balanced growth, *R. eutropha* JMP134 first and foremost replicates and safeguards the genetic information. Under conditions of unbalanced nutrient composition and suddenly changing availability, cells respond with overflow metabolism or, under yet poorer conditions, with dormancy. This occurs independently of the substrate. However, the nature of the substrate determines the growth rate by initiation of uncoupled DNA synthesis (in the case of rich substrate) or by causing a delayed generation time (on poor substrate). The adaptive response of *R. eutropha* JMP134 to these contrasting situations is qualitatively similar but, depending upon the nature of the available substrates, it can be quantitatively very different.

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