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Entry of *Escherichia coli* into Stationary Phase Is Indicated by Endogenous and Exogenous Accumulation of Nucleobases

URSULA RINAS,* KARSTEN HELLMUTH, RUIJUAN KANG,†
ANKE SEEGER, AND HEINRICH SCHLIEKER

Biochemical Engineering Division, GBF National Research Center for Biotechnology, 38124 Braunschweig, Germany

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Endogenous and exogenous accumulation of nucleobases was observed when *Escherichia coli* entered the stationary phase. The onset of the stationary phase was accompanied by excretion of uracil and xanthine. Except for uracil and xanthine, other nucleobases (except for minor amounts of hypoxanthine), nucleosides, and nucleotides (except for cyclic AMP) were not detected in significant amounts in the culture medium. In addition to exogenous accumulation of nucleobases, stationary-phase cells increased the endogenous concentrations of free nucleobases. In contrast to extracellular nucleobases, hypoxanthine was the dominating intracellular nucleobase and xanthine was present only in minor concentrations inside the cells. Excretion of nucleobases was always connected to declining growth rates. It was observed in response to entry into the stationary phase independent of the initial cause of the cessation of cell growth (e.g., starvation for essential nutrients). In addition, transient accumulation of exogenous nucleobases was observed during perturbations of balanced growth conditions such as energy source downshifts. The nucleobases uracil and xanthine are the final breakdown products of pyrimidine (uracil and cytosine) and purine (adenine and guanine) bases, respectively. Hypoxanthine is the primary degradation product of adenine, which is further oxidized to xanthine. The endogenous and exogenous accumulation of these nucleobases in response to entry into the stationary phase is attributed to degradation of rRNA.

To survive periods of prolonged starvation, cells need to adapt from nutrient-rich to nutrient-poor conditions (17). *Escherichia coli* is a nondifferentiating bacterium which does not produce endospores in response to starvation conditions. Nevertheless, the transition from the exponential to the stationary phase is accompanied by drastic changes in cellular morphology and physiology (7, 12, 18, 19, 28, 43). Cells develop a general resistance to stress (27), and despite a general reduction in protein synthesis, a set of unique proteins is synthesized when cells enter the stationary phase (10, 11, 35, 42). During glucose-starvation-induced entry into the stationary phase, endogenous levels of cyclic AMP (cAMP) increase instantaneously, with the majority of cAMP being excreted into the medium (3, 25, 37). It has been suggested that the release of cAMP into the growth medium may play a regulatory role (3, 25, 37). However, cAMP-dependent starvation proteins do not appear to be essential for survival of starvation conditions, and strains carrying deletions in the genes encoding adenylate synthase (Δ*cya*) or the cAMP receptor protein (Δ*crp*) survive starvation as well as their wild-type parents (42). In addition, it has been shown that *cya* and *crp* mutants exhibit increased, multiple stress resistance compared with their wild-type parents, including increased resistance to sublethal heat and hypoosmotic stress (21).

Recently, the *rpoS* gene (*katF*) has been identified as encoding a putative stationary-phase sigma factor (σ^s) which is involved in the transcriptional regulation of stationary-phase inducible genes and development of starvation-mediated general resistance in *E. coli* (22, 30). A complex network of transcriptional and posttranscriptional control mechanisms is involved in regulation of σ^s levels, and there are indications that at least five different signals (cAMP, ppGpp, a cell density signal, an osmotic signal, and a starvation signal) are involved in the processes that regulate *rpoS* expression (9, 23, 24, 29). Expression of *rpoS* is induced by a dialyzable, heat-stable metabolite(s) present in spent medium (32). Attempts to identify the metabolite by adding fermentation by-products to fresh medium have been unsuccessful (32). More recently, it has been hypothesized that the cellular content of UDP-glucose may serve as an internal signal controlling the expression of σ^s-dependent genes and of σ^s itself (2). However, the molecular signal triggering the stationary-phase response is still not identified.

In this study, we describe the endogenous and exogenous accumulation of free nucleobases by *E. coli* in response to entry into the stationary phase. Nucleobases are normally not present intra- or extracellularly as free bases or nucleosides; they are found almost exclusively as nucleotides (33).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains TG1, TG2, JM101, JM105, JM109, DH_1, and DH_5 were described previously (39). *E. coli* K-12 strain MC4100 was described by Casadaban (4). *E. coli* K-12 strain MG1655 was from B. Bachmann.

Shake flask cultivation. Cells were grown on glucose minimal medium (20) in shake flask cultures (10 ml of medium in 100-ml baffled Erlenmeyer flasks) for investigation of extracellular accumulation of uracil by different *E. coli* strains. Cultures were inoculated with an overnight culture (5-ml culture in a test tube) to an initial optical density (OD) of 0.1 and incubated on a rotary shaker at 37°C for 48 h.

Batch and continuous cultivation. Batch and continuous cultivations were carried out at 28°C in a 3.7-liter bioreactor (model KLF2000; Bioengineering, Wald, Switzerland) with a working volume of 2.5 liters. The pH was kept constant at pH 6.6 by using 10% NaOH for pH control. The aeration rate was 0.5 vol/vol/min at 1,500 rpm. Dissolved oxygen concentrations were analyzed with a polarographic electrode (Ingold; Mettler Toledo Prozessanalytik GmbH, Steinbach, Germany). The concentrations of oxygen and carbon dioxide in the exhaust gas were determined by paramagnetic and infrared gas analysis systems, respec-

* Corresponding author. Mailing address: Biochemical Engineering Division, GBF National Research Center for Biotechnology, Mascheroder Weg 1, 38124 Braunschweig, Germany. Phone: 0049-531-6181-126. Fax: 0049-531-6181-111. Electronic mail address: URI@gbf-braunschweig.de.

† Present address: Institute of Chemical Metallurgy, Chinese Academy of Sciences, Beijing 100080, China.

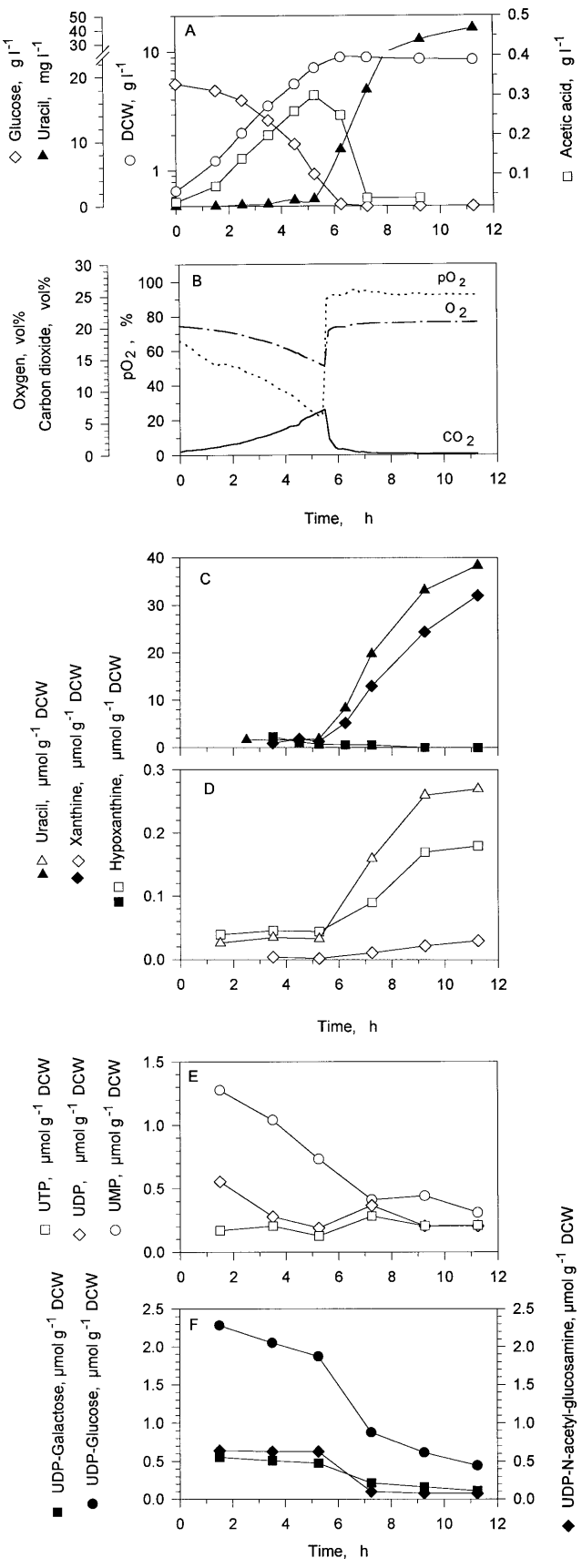

FIG. 1. Batch cultivation of *E. coli* TG1 with minimal medium with glucose as the carbon source. The time courses of dry cell weight, glucose, acetic acid, and uracil concentrations in the cell-free culture medium (A), dissolved oxygen concentration (pO_2, percent air saturation) and oxygen and carbon dioxide concentrations in the exhaust gas (B), specific concentrations of extracellular uracil, xanthine, and hypoxanthine (C), specific concentrations of intracellular uracil, xanthine, and hypoxanthine (D), specific intracellular concentrations of UTP, UDP and UMP (E), and specific intracellular concentrations of UDP-glucose, UDP-galactose, and UDP-*N*-acetyl-glucosamine during batch growth are shown.

tively (Maihak, Hamburg, Germany). The medium composition for precultures and for batch cultivations was as follows: glucose (20 $g \cdot liter^{-1}$), $(NH_4)_2SO_4$ (16 $g \cdot liter^{-1}$), KH_2PO_4 (2.7 $g \cdot liter^{-1}$), $(NH_4)_2HPO_4$ (0.8 $g \cdot liter^{-1}$), $MgSO_4 \cdot 7H_2O$ (1 $g \cdot liter^{-1}$), citric acid-H_2O (0.35 $g \cdot liter^{-1}$), EDTA (1.7 $mg \cdot liter^{-1}$), Fe(III) citrate-H_2O (12 $mg \cdot liter^{-1}$), $CoCl_2 \cdot 6H_2O$ (0.5 $mg \cdot liter^{-1}$), $MnCl_2 \cdot 4H_2O$ (3 $mg \cdot liter^{-1}$), $CuCl_2 \cdot 2H_2O$ (0.3 $mg \cdot liter^{-1}$), $Na_2MoO_4 \cdot 2H_2O$ (0.5 $mg \cdot liter^{-1}$), $Zn(CH_3COO)_2 \cdot 2H_2O$ (1.6 $mg \cdot liter^{-1}$), H_3BO_3 (0.6 $mg \cdot liter^{-1}$), thiamine-HCl (4 $mg \cdot liter^{-1}$) and, if necessary, the antifoam reagent Desmophen (0.1 $ml \cdot liter^{-1}$). Glucose and $MgSO_4 \cdot 7H_2O$ were sterilized separately at 121°C. Thiamine was sterilized by filtration. For continuous cultivations, the same medium was used as that described above except that the concentrations of glucose and $(NH_4)_2SO_4$ were 10 and 8 $g \cdot liter^{-1}$, respectively. Precultures (100 ml medium in 1,000-ml shake flasks) were grown overnight on a rotary shaker at 30°C.

Fed-batch cultivation. The fed-batch cultivation of *E. coli* TG1 with glycerol as the carbon source was carried out essentially as described by Korz et al. (20). The cultivation temperature was 28°C, and the dissolved oxygen concentration was maintained at 40% of air saturation. The preparation of the batch medium was done as described, except that the initial concentration of glycerol was 46.5 $g \cdot liter^{-1}$. The feeding protocol was modified to prevent phosphate-limiting conditions and to minimize dilution of the culture by the use of more highly concentrated feeding solutions. The carbon source feeding solution was prepared by the addition of 50.15 g of $MgSO_4 \cdot 7H_2O$ to 2 liters of glycerol (99%) and sterilized for 30 min at 121°C (density of the glycerol feeding solution, 1.26 $g \cdot ml^{-1}$). Exponential glycerol feeding allowing carbon-limited cell growth at desired specific growth rates was carried out as described previously (20); however, carbon source feeding was adjusted to reduce stepwise the specific growth rate during the fed-batch process (details are specified in Results). Feeding solutions containing phosphate and trace elements were prepared separately. The phosphate feeding solution was composed of KH_2PO_4 (327 $g \cdot liter^{-1}$) and $(NH_4)_2HPO_4$ (106 $g \cdot liter^{-1}$). The composition of the trace element feeding solution was as follows: EDTA (2.32 $g \cdot liter^{-1}$), $CoCl_2 \cdot 6H_2O$ (0.296 $g \cdot liter^{-1}$), $MnCl_2 \cdot 4H_2O$ (0.527 $g \cdot liter^{-1}$), $CuCl_2 \cdot 2H_2O$ (0.601 $g \cdot liter^{-1}$), H_3BO_3 (0.83 $g \cdot liter^{-1}$), $Na_2MoO_4 \cdot 2H_2O$ (0.691 $g \cdot liter^{-1}$), $Zn(CH_3COO)_2 \cdot 2H_2O$ (2.212 $g \cdot liter^{-1}$), Fe(III) citrate (0.538 $g \cdot liter^{-1}$), and thiamine (0.146 $g \cdot liter^{-1}$). Stock solutions of EDTA, trace elements, and thiamine were prepared as described previously (20). Feeding of phosphate and trace element solutions (linear rate, each 9 $ml \cdot h^{-1}$) was started when the cell density reached 75 g of dry cell weight (DCW) $liter^{-1}$. Aqueous ammonia (25% [wt/wt]), which was always used for pH control (pH 6.9), also served as the nitrogen source.

Analytical methods. Cell growth was monitored by measurement of the OD at a wavelength of 600 nm (Novaspec II; Pharmacia LKB, Freiburg, Germany; 1 OD unit at 600 nm corresponds to 0.52 g of DCW $liter^{-1}$). In addition, DCWs were determined from 1-ml aliquots of culture broth collected in balanced 1.5-ml centrifugation tubes. Cell pellets were collected by centrifugation for 3 min at 3,300 × *g*, resuspended in distilled water, centrifuged again, and dried at 40°C under vacuum until constancy of weight was achieved.

Concentrations of glucose, glycerol, acetic acid, and uracil were determined by high-performance liquid chromatography (HPLC) analysis with an Aminex HXP-87H column for separation and UV and refractive index detectors for detection. Sulfuric acid (5 mM) was used as the mobile phase (flow rate, 0.5 $ml \cdot min^{-1}$).

An ammonium electrode (type Orion 95-12; Colora, Lorch, Germany) was employed to analyze the concentrations of ammonium. The concentration of phosphate was analyzed by a modified procedure described in the *German Standard Methods* (45).

Samples for analysis of intracellular compounds were prepared as follows. Five-milliliter volumes of culture samples were transferred directly from the bioreactor into precooled centrifugation tubes (15-ml Falcon tubes on ice-salt mixture, −20°C) and centrifuged at 4,000 × *g* for 15 min (4°C). The supernatant was discarded, and the cell pellet was processed as described previously (38). Cell extracts for each sample were prepared in duplicate. Soluble cell extracts were stored at −70°C, and HPLC analysis of nucleobases, nucleotides, and sugar nucleotides was carried out as described previously (38).

RESULTS

Exogenous and endogenous accumulation of nucleobases by *E. coli* TG1 in response to entry into the stationary phase. *E. coli* TG1 was grown on glucose minimal medium in a batch process as described in Materials and Methods (Fig. 1). After exhaustion of glucose, excretion of nucleobases occurred (Fig. 1A; only exogenous concentrations of uracil are shown). Prior to glucose depletion, only minor concentrations of nucleobases were detected in the culture supernatant. An increased release of nucleobases was observed after exhaustion of easily utilizable glucose but prior to the exhaustion of the metabolic byproduct acetic acid (Fig. 1A). The onset of exogenous nucleobase accumulation occurred concomitantly with the reduction of growth rate and entry into the stationary phase. Entry into the stationary phase was also reflected in the dissolved oxygen concentration and the concentrations of oxygen and carbon dioxide in the exhaust gas (Fig. 1B). Entry into the stationary phase was caused by carbon starvation, oxygen limitation was not observed (Fig. 1B), and phosphate- and nitrogen-limiting conditions were not detected (data not shown). In addition to exogenous uracil accumulation, excretion of xanthine (and minor amounts of hypoxanthine) occurred in response to entry into the stationary phase (Fig. 1C). Other nucleobases, nucleosides, nucleotides (except for cAMP), and sugar nucleotides were not detected in significant amounts in the culture medium after entry into the stationary phase (data not shown). The nucleobases uracil and xanthine are the final breakdown products of pyrimidine (uracil and cytosine) and purine (adenine and guanine) bases, respectively. In addition to exogenous accumulation of nucleobases, intracellular concentrations of uracil and hypoxanthine increased in response to entry into the stationary phase (Fig. 1D). In contrast to exogenous nucleobases, hypoxanthine was the dominating intracellular nucleobase and xanthine was present only in minor amounts inside the cells. Hypoxanthine is the primary degradation product of adenine, which is further oxidized to xanthine. More than 99% of the entire free uracil (and xanthine) was found in the culture medium during the stationary phase. Analysis of intracellular concentrations of low-molecular-weight (LMW) uracil-containing nucleotides and sugar nucleotides revealed that free uracil could not originate solely from breakdown of LMW compounds. The decrease in the intracellular specific concentrations of LMW uracil-containing metabolites during transition from growth to nongrowth conditions is too small to account for the entire free uracil found intra- and extracellularly during the late stationary phase (Fig. 1C to F; Table 1). In addition, total breakdown of unstable RNA (mRNA) and release of free uracil originating from uracil- and, by deamination, from cytosine-containing mRNA pyrimidine nucleotides is not sufficient to account for the total free uracil accumulated by *E. coli* during the stationary phase (Table 1). Endogenous and exogenous accumulation of free uracil must result from breakdown of stable RNA (tRNA or rRNA).

Excretion of nucleobases (only uracil was tested) during the stationary phase was also observed when another carbon source was used (e.g., glycerol) or when cells were grown on complex medium (Luria broth; data not shown).

Exogenous accumulation of nucleobases during steady-state carbon-limited growth. Extracellular concentrations of uracil (excretion of other nucleobases was not tested) during steady-state growth of *E. coli* TG1 in glucose-limited chemostat cultures did not exceed 1 mg · liter^{-1} (0.2 mg · g of DCW^{-1}) at dilution rates ranging from 0.1 to 0.4 h^{-1} (data not shown).

Exogenous accumulation of nucleobases during the stationary phase is not specific to *E. coli* TG1. Excretion of nucleobases was not unique to *E. coli* TG1. All other *E. coli* strains tested (listed in Materials and Methods) excreted uracil (excretion of other nucleobases was not tested), although in different amounts, into the culture fluid during the stationary phase. In shake flask cultures, up to 60 mg of uracil liter^{-1} corresponding to 16 to 20 mg · g of DCW^{-1} accumulated in the medium within 48 h of cultivation (data not shown). Excretion of uracil was observed in both stringent and relaxed strains.

TABLE 1. Specific concentrations of intra- and extracellular free uracil and intracellular uracil present in LMW (sugar) nucleotides and RNA during the exponential and late stationary phases

Compound	Concn (μmol of uracil · g of DCW^{-1}) in:	
	Exponential growth phase	Late stationary phase
Extracellular free uracil	1.70	38.00
Intracellular free uracil	0.0310 ± 0.004	0.24 ± 0.004
Intracellular uracil present in LMW (sugar) nucleotides	Σ 5.02	Σ 1.53
UXP (UTP + UDP + UMP)	1.75 ± 0.25	0.81 ± 0.07
UDP-glucose	2.14 ± 0.14	0.51 ± 0.06
UDP-galactose	0.51 ± 0.04	0.14 ± 0.03
UDP-*N*-acetyl-glucosamine	0.62 ± 0.02	0.07 ± 0.01
UMP in total RNA[a]	136.0	
UMP in mRNA[a] (mRNA = 4% of total RNA)	5.4	
CMP in mRNA[a] (mRNA = 4% of total RNA)	5.0	

[a] Data were obtained from Ingraham et al. (13).

Exogenous accumulation of nucleobases as a sensitive indicator of growth perturbations in high-cell-density cultures of *E. coli*. Cessation of cell growth in high-cell-density carbon-limited fed-batch cultures of *E. coli* TG1 was attributed to phosphate starvation conditions (20). When cells reached conditions of phosphate starvation in high-cell-density cultures, intensive excretion of uracil (excretion of other nucleobases was not tested) was observed (data not shown). To prevent phosphate-limiting conditions, a modified fed-batch procedure was developed. In addition to carbon source feeding (glycerol, including $MgSO_4$), supplementary feeding of phosphate [KH_2PO_4 or $(NH_4)_2HPO_4$)] and trace elements was carried out (see Materials and Methods) (Fig. 2). Cells were grown under carbon-limiting conditions, and the exponential feeding rate was adjusted in such a way that the specific growth rate was reduced stepwise (Fig. 2A). After feed rate reduction (μ_{set} = 0.1 to 0.06 h^{-1} and μ_{set} = 0.06 to 0.04 h^{-1}), exogenous accumulation of uracil (excretion of other nucleobases was not tested) followed by subsequent reassimilation occurred (Fig. 2B). When a final cell density of 165 g of DCW · liter^{-1} (OD at 600 nm, ≈320) was reached (Fig. 2A and C), cell growth stopped and excretion of uracil was observed (Fig. 2B). Final cessation of cell growth occurred irrespective of the continuing addition of feed medium [glycerol, $MgSO_4$, KH_2PO_4 and $(NH_4)_2HPO_4$, and trace elements] and was accompanied by increased formation of acetic acid and a reduction in the respiratory activity reflected in decreasing carbon dioxide concentrations in the off-gas (Fig. 2B and C, respectively). We could not identify any limiting substrate at the end of the high-cell-density process. The dissolved oxygen concentration was maintained at 40% of air saturation. Phosphate and ammonium concentrations were not limiting (Fig. 2D). Final cessation of cell growth was not attributable to starvation conditions. Arrest of cell growth may have occurred because of extremely high cell densities.

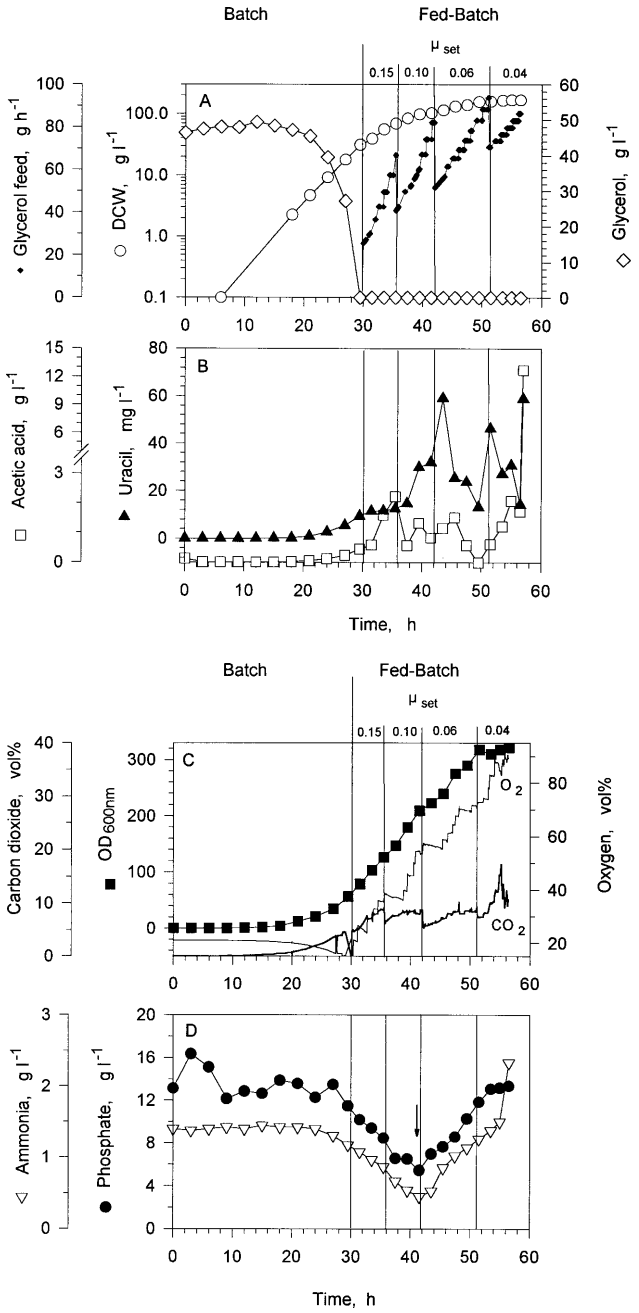

FIG. 2. High-cell-density cultivation of *E. coli* TG1 with glycerol as the carbon source. After unlimited growth during batch mode at 28°C (μ_{max} = 0.29 h^{-1}), fed-batch mode was started with a desired specific growth rate of μ_{set} = 0.15 h^{-1}. The exponential feeding profile was then adjusted to allow a stepwise reduction of the specific growth rate as indicated. (A) Time course of dry cell weight, mass flow of glycerol into the bioreactor, and glycerol concentration in the cell-free culture medium. (B) time course of uracil and acetic acid concentrations in the cell-free culture medium. (C) time course of OD at 600 nm and concentrations of oxygen and carbon dioxide in the exhaust gas. Increasing concentrations of oxygen in the exhaust gas during the fed-batch phase are caused by blending inlet air with pure oxygen to maintain the dissolved oxygen concentrations at 40% of air saturation (20). (D) Time course of ammonia (NH_3) and phosphate (PO_4^{3-}) concentrations in the cell-free culture medium. The arrow indicates the onset of phosphate and trace element feeding.

DISCUSSION

The presence of uracil among the free nucleobases accumulated during the stationary phase suggests that the free nucleobases may originate from degradation of RNA since uracil is the nucleobase found exclusively in RNA, not in DNA. Uracil is present in LMW nucleotides such as UMP, UDP, and UTP, in UDP sugars, and in RNA. Only a minor part of uracil is found in LMW nucleotides and sugar nucleotides; around 95% of the cellular uracil is present in RNA (33). The difference of uracil-containing LMW metabolites such as UDP sugars and (uracil) nucleotides present intracellularly prior to and at the end of the stationary phase during batch cultivation revealed that the entire free uracil found intra- and extracellularly cannot result simply from decreasing concentrations of these metabolites during transition from growth to nongrowth conditions (Table 1). In addition, total breakdown of unstable RNA (mRNA) and release of free uracil originating from uracil and, by deamination, from cytosine-containing mRNA pyrimidine nucleotides is not sufficient to account for the total free uracil accumulated by *E. coli* during the stationary phase (Table 1). Endogenous and exogenous accumulation of free uracil must result from breakdown of stable RNA (tRNA and rRNA).

Assuming that only 5% of the total RNA is represented in mRNA and 15% is represented in tRNA, the main part of uracil is found in rRNA (13, 34). rRNA and tRNA are considered in general to be stable molecules; however, turnover of newly made rRNA not yet incorporated into ribosomes increases with decreasing growth rates (8) as well as during the transition of energy source downshifts (31). In addition, when *E. coli* is starved for essential nutrients, extensive degradation of rRNA occurs (1, 7, 14, 15, 26, 36). In contrast to rRNA, tRNA continued to be preserved upon prolonged starvation of *E. coli* (6). Incorporation of exogenously supplied radioactive uracil into rRNA of exponentially growing *E. coli* and subsequent exposure of cells to starvation conditions revealed that uracil previously incorporated into acid-insoluble material was released into acid-soluble material during starvation (16). Identification of radioactive acid-soluble rRNA degradation products indicated that pyrimidine bases excreted into the culture medium constitute the majority of these degradation products (5, 16). Altogether, it is concluded that exogenous and endogenous accumulation of uracil [and (hypo)xanthine] in response to entry into the stationary phase or to perturbations of balanced growth conditions such as energy source downshifts originates from breakdown of rRNA. Uracil and xanthine are the final breakdown products of pyrimidine (uracil and cytosine) and purine (adenine and guanine) bases, respectively, present in RNA. Hypoxanthine is the primary degradation product of adenine, which is further oxidized to xanthine.

Even though excessive loss of ribosomes may be a major cause of cell death (6), the cellular ability to degrade rRNA is closely connected to the cellular fitness to survive starvation conditions (14). The role of ribosomes or ribosomal breakdown products as primary sensors of stimulus-response networks such as heat and cold shock and the stringent response has been discussed, and it has been hypothesized that ribosomes may serve as primary sensors of conditions that invoke a stringent response either by producing the nucleotide ppGpp or by the state of the ribosome itself (44). Although we do not have any experimental evidence that rRNA or ribosomal breakdown products are involved in triggering the stationary-phase response, the data presented show a correlation between these two phenomena and it does not seem unreasonable to speculate about the role of rRNA or ribosomal breakdown products as sensors of conditions that invoke the stationary-phase response. Excretion of nucleobases and, therefore, degradation of rRNA always occurred in response to declining growth rates. Accumulation of nucleobases was observed in

response to entry into the stationary phase and was independent of the initial cause responsible for cessation of cell growth (e.g., starvation for essential nutrients). In addition, transient accumulation of exogenous nucleobases was observed during perturbations of balanced growth conditions such as energy source downshifts, demonstrating the correlation of exogenous nucleobase accumulation to conditions resulting in declining growth rates. The fact that a dialyzable (hence, a LMW compound) and heat-stable metabolite(s) present in spent and not in fresh medium can induce the expression of *rpoS* (32, 40) makes ribosomal breakdown products such as free nucleobases suitable candidates for the stationary-phase-response-inducing metabolite(s). Although uracil, xanthine (and minor concentrations of hypoxanthine), and cAMP were the dominant substances released during glucose starvation-induced entry into the stationary phase, it is not excluded that several minor peaks corresponding to (to-date) unidentified compounds appearing in HPLC of stationary-phase medium may represent the stationary-phase-response-inducing metabolite(s) (data not shown). Attempts to identify the inducing metabolite by adding fermentation by-products to fresh medium have been unsuccessful (32), and the observation that weak acids such as acetic acid can induce transcription of *rpoS* is probably correlated to the growth rate decrease caused by the addition of these substances (41).

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