

# Specific growth rate and not cell density controls the general stress response in *Escherichia coli*

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In batch cultures of *Escherichia coli*, the intracellular concentration of the general stress response sigma factor RpoS typically increases during the transition from the exponential to the stationary growth phase. However, because this transition is accompanied by complex physico-chemical and biological changes, which signals predominantly elicit this induction is still the subject of debate. Careful design of the growth environment in chemostat and batch cultures allowed the separate study of individual factors affecting RpoS. Specific growth rate, and not cell density or the nature of the growth-limiting nutrient, controlled RpoS expression and RpoS-dependent hydroperoxidase activity. Furthermore, it was demonstrated that the standard *E. coli* minimal medium A (MMA) is not suitable for high-cell-density cultivation because it lacks trace elements. Previously reported cell-density effects in chemostat cultures of *E. coli* can be explained by a hidden, secondary nutrient limitation, which points to the importance of medium design and appropriate experimental set-up for studying cell-density effects.

## INTRODUCTION

The  $\sigma^s$  (RpoS) subunit of RNA polymerase enables *Escherichia coli* rapidly to adapt to various stress conditions by redirecting mRNA synthesis. RpoS is a key factor for starvation survival (Lange & Hengge-Aronis, 1991) and has become a paradigm for global regulatory networks in bacteria since its recognition as an alternative sigma factor (Mulvey & Loewen, 1989; Lange & Hengge-Aronis, 1991).

In batch cultures, intracellular levels of RpoS typically increase during the transition from the exponential phase to the stationary phase (Gentry *et al.*, 1993; Jishage *et al.*, 1996; Lange & Hengge-Aronis, 1994), making stationary-phase cells broadly stress-resistant. What triggers this increase has been the subject of much debate, but it is clear that RpoS is controlled in a complex manner at the transcriptional, translational and protein-stability levels (Lange & Hengge-Aronis, 1994; Loewen *et al.*, 1993; McCann *et al.*, 1993; for an overview see Hengge-Aronis, 2002). Different environmental signals have been shown to induce elevated intracellular RpoS levels, among them reduction in growth rate (Notley & Ferenci, 1996; Teich *et al.*, 1999), high osmolarity (Muffler *et al.*, 1996), heat shock (Muffler *et al.*, 1997), temperature downshift (Sledjeski *et al.*, 1996) and acidic pH (Bearson *et al.*, 1996).

Cell density has also been suggested to be an important signal for RpoS expression (Lange & Hengge-Aronis, 1994).

Cell-density effects are generally thought to be mediated by external signal molecules that affect gene expression when they reach a certain threshold concentration, a phenomenon called quorum sensing (Fuqua *et al.*, 1994). This was first described for *Vibrio fischeri*, where homoserine lactone serves as signal molecule (autoinducer) for luminescence (Nealson, 1977). Under certain conditions, *E. coli* excretes into the medium a compound, referred to as autoinducer 2, which is able to induce luminescence in *Vibrio harveyi* (Surette & Bassler, 1998; Surette *et al.*, 1999). Therefore, quorum sensing has been postulated as a regulatory mechanism in *E. coli* and *Salmonella typhimurium* (Surette & Bassler, 1998; Surette *et al.*, 1999; Sperandio *et al.*, 2001).

Conflicting data exist with regard to a quorum-sensing-mediated regulation of RpoS (reviewed by Hengge-Aronis, 2002). Intracellular RpoS concentrations increased when cell density rose from 0.09 to 0.5 g dry cell weight per litre (g DCW l<sup>-1</sup>) in chemostat cultures of *E. coli* strain MC4100 at a constant dilution rate (*D*) of 0.3 h<sup>-1</sup> (Liu *et al.*, 2000). In contrast, *rpoS* mRNA levels were constant during an increase in cell density from 10 to 90 g DCW l<sup>-1</sup> in fed batch cultures of *E. coli* strain W3110 (Yoon *et al.*, 2003). *RpoS* transcription in *Pseudomonas aeruginosa* PAO1 was dependent on functional LasR and RhIR, activators that respond to *N*-acylhomoserine lactone (AHL) signal molecules accumulating at high cell density (Latifi *et al.*, 1996). However, overexpression of RelA allowed AHL production and RpoS expression at low cell density also (van Delden *et al.*, 2001).

Abbreviations: AHL, *N*-acylhomoserine lactone; *D*, dilution rate; DCW, dry cell weight; HPI, hydroperoxidase I; HPII, hydroperoxidase II.

In standard batch cultures, it is difficult to attribute an observed response exclusively to quorum sensing. During the transition from unrestricted growth to the stationary phase, not only does cell density change, but also specific growth-rate, extracellular carbon-source availability, metabolite concentrations (Wanner & Egli, 1990) and, in the case of LB, amino-acid availability. These complex changes in turn affect the level of intracellular signal molecules like cAMP and (p)ppGpp, and RpoS expression has been reported to be influenced by (p)ppGpp (Gentry *et al.*, 1993; Lange *et al.*, 1995), cAMP (Lange & Hengge-Aronis, 1991; Lange & Hengge-Aronis, 1994) and acetate (Schellhorn & Stones, 1992), which is the main metabolite excreted by *E. coli* in aerobic batch culture. Chemostat cultivation offers an elegant way out of the complex situation in batch culture (Liu *et al.*, 2000) because the specific growth rate (which equals  $D$  under steady-state conditions) and physico-chemical factors can be kept constant, while single parameters like cell density or the nature of the limiting nutrient are varied (Pirt, 1975).

The aim of the present study was to assess the contribution of individual environmental and physiological factors to the increase in RpoS seen in batch and certain chemostat cultures of *E. coli*. For this, the effects of different steady-state cell densities and different types of limitation on RpoS were studied in chemostat cultures growing at similar specific growth rates. In another set of experiments, cell density was kept low while specific growth rate was varied.

We demonstrate that neither cell density nor the type of limitation have an effect on RpoS expression, whereas specific growth rate exerts a strong control. Furthermore, we have found that the standard *E. coli* minimal medium A (MMA) is unsuitable for cultures with cell densities higher than  $0.5 \text{ g DCW l}^{-1}$  because it lacks trace elements. Hence, earlier observations of cell-density effects in *E. coli* are most likely due to a hidden shift in the growth-limiting nutrient.

## METHODS

**Bacterial strains.** To exclude unwanted effects of mutations on RpoS expression, wild-type *E. coli* K-12 MG 1655 [genotype  $F^- \lambda^- rph1$ ] was used for most experiments (obtained from the culture collection of the late P. Postma, Amsterdam). Other strains used in this study were an *rpoS13::Tn10* allele of MG 1655 (Wick *et al.*, 2002) and BW2952, an MC4100 derivative containing a *malG-lacZ* fusion [genotype  $F^- \text{araD139 } \Delta(\text{argF-lac})\text{U169 rpsL150 deoCa relA1 thiA ptsF25 flbB5301 rbsR malG}::\lambda\text{placMu55 } \phi(\text{malG}::\text{lacZ})$ ] (Notley-McRobb *et al.*, 2002).

**Growth media and their design.** Mineral medium allowing carbon-limited cultivation up to a glucose concentration of  $4.0 \text{ g l}^{-1}$  was designed, based on the average composition of bacterial biomass as previously described (Egli, 2000; Pirt, 1975). Basal medium for carbon-limited batch cultivation (medium  $C_{\text{lim}}\text{Ba}$ ) consisted of  $12.8 \text{ g Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ,  $3.0 \text{ g KH}_2\text{PO}_4$  and  $1.77 \text{ g (NH}_4)_2\text{SO}_4$  per litre. The following salts and trace elements were added as a 100-fold concentrated solution after autoclaving (final concentrations are given):  $130 \text{ mg MgCl}_2 \cdot 6\text{H}_2\text{O l}^{-1}$ ;  $80 \text{ mg CaCO}_3 \text{ l}^{-1}$ ,  $77 \text{ mg FeCl}_3 \cdot 6\text{H}_2\text{O l}^{-1}$ ,  $11 \text{ mg MnCl}_2 \cdot 4\text{H}_2\text{O l}^{-1}$ ,  $1.5 \text{ mg}$

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O l}^{-1}$ ,  $1.3 \text{ mg CoCl}_2 \cdot 6\text{H}_2\text{O l}^{-1}$ ,  $4 \text{ mg ZnO l}^{-1}$ ,  $1.2 \text{ mg H}_3\text{BO}_3 \text{ l}^{-1}$ ,  $10 \text{ mg NaMoO}_4 \cdot 2\text{H}_2\text{O l}^{-1}$  and  $790 \text{ mg EDTA Na}_4 \cdot 2\text{H}_2\text{O l}^{-1}$  (equimolar to di- and trivalent cations). Glucose was always autoclaved separately and added after cooling. For high-growth-rate batch culture,  $2 \text{ g l}^{-1}$  Casamino Acids and a mixture of 11 vitamins were added to medium  $C_{\text{lim}}\text{Ba}$  at the following final concentrations: biotin and folic acid  $20 \mu\text{g l}^{-1}$ ; pyridoxine  $100 \mu\text{g l}^{-1}$ ; thiamin, riboflavin, nicotinic acid, vitamin B12, calcium D-pantothenate, *p*-aminobenzoic acid, lipoic acid and nicotinamide  $50 \mu\text{g l}^{-1}$ .

Medium for carbon-limited chemostat cultivation (medium  $C_{\text{lim}}\text{Ch}$ ) consisted of  $2.72 \text{ g KH}_2\text{PO}_4$ ,  $2.3 \text{ g NH}_4\text{Cl}$ ,  $1.4 \text{ g (NH}_4)_2\text{SO}_4$ ,  $0.1 \text{ ml concentrated H}_2\text{SO}_4$  and  $0.15 \text{ ml silicone anti-foam (Fluka) per litre}$ . Trace elements in the concentrations mentioned above could be added before autoclaving because the medium pH was around 4.0, which prevents salt precipitation.

For iron-limited chemostat cultivation (medium  $\text{Fe}_{\text{lim}}\text{Ch}$ ), iron was omitted from the trace element solution. For nitrogen-limited chemostat cultivation (medium  $\text{N}_{\text{lim}}\text{Ch}$ ), the ammonium supply was reduced to  $0.21 \text{ g NH}_4\text{Cl l}^{-1}$  (ammonium sulfate was omitted) and concentrated  $\text{H}_2\text{SO}_4$  was increased to  $0.15 \text{ ml l}^{-1}$  to guarantee surplus sulphur supply. Luria-Bertani broth (LB) contained  $10 \text{ g tryptone}$ ,  $5 \text{ g yeast extract}$  and  $10 \text{ g NaCl per litre}$  (Miller, 1972). Minimal medium A (MMA) consisted of  $10.5 \text{ g K}_2\text{HPO}_4$ ,  $4.5 \text{ g KH}_2\text{PO}_4$ ,  $0.5 \text{ g sodium citrate}$  and  $0.25 \text{ g MgSO}_4 \cdot 7\text{H}_2\text{O per litre}$  (Miller, 1972). To avoid nitrogen limitation at a high-glucose-feed concentration,  $(\text{NH}_4)_2\text{SO}_4$  concentration was increased from  $1.0$  to  $4.3 \text{ g l}^{-1}$ . Thiamin was added at a concentration of  $1 \text{ mg l}^{-1}$ , if required (e.g. for strain BW2952). For direct comparison to medium  $C_{\text{lim}}\text{Ch}$ , modified MMA was used that contained (per litre):  $2.72 \text{ g KH}_2\text{PO}_4$ ,  $2.3 \text{ g NH}_4\text{Cl}$ ,  $1.4 \text{ g (NH}_4)_2\text{SO}_4$ ,  $0.1 \text{ ml H}_2\text{SO}_4$ ,  $0.42 \text{ g citric acid monohydrate}$ ,  $0.25 \text{ g MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $0.15 \text{ ml silicone anti-foam}$ . Viable cell numbers were determined by plating on tryptone glucose yeast extract agar (TGY; Biolife). Nanopure water and high-purity chemicals were used for all solutions and media.

**Cultivation conditions.** For batch cultures, bacteria were incubated at  $37^\circ\text{C}$  in electromagnetically stirred Erlenmeyer flasks. For continuous culture, computer-controlled glass and stainless steel bioreactors (MBR, Wetzikon, Switzerland) were used. pH was kept at  $7.0 \pm 0.1$  by automatic addition of a solution of  $0.5 \text{ M NaOH}$  and  $0.5 \text{ M KOH}$  and the temperature was set at  $37 \pm 0.1^\circ\text{C}$ . Oxygen concentration was kept between 95 and 100% of air saturation at  $37^\circ\text{C}$ , and the stirrer speed was set to  $800 \text{ r.p.m.}$

Some *E. coli* strains quickly acquire mutations in *rpoS* when cultivated in carbon-limited chemostats (Notley-McRobb *et al.*, 2002). To avoid the accumulation of mutations in the populations investigated, a new preculture was prepared for each individual batch or chemostat experiment from the same cryo-vial stored at  $-80^\circ\text{C}$ . Colonies grown on TGY agar for 15–18 h at  $37^\circ\text{C}$  were transferred to 5 ml of either complex or mineral medium used later in the experiments. After 3 h incubation at  $37^\circ\text{C}$ , 1–2 ml of the culture was transferred into 100 ml medium in Erlenmeyer flasks. This culture was used as inoculum for the bioreactors. Batch experiments were conducted in three parallel Erlenmeyer flasks, containing pre-warmed medium, that were inoculated with exponentially growing cells from 5 ml cultures. For batch experiments with glucose mineral medium, cultures were grown overnight before using them as inoculum. Dilutions were repeated as often as necessary to keep cultures in exponential phase. In chemostat cultures, steady-state is defined as constant optical density, and this was always reached after 5–10 volume changes. Chemostats were sampled 30–50 h after inoculation, which corresponds to 13–22 generations at a dilution rate of  $0.3 \text{ h}^{-1}$ . Purity of chemostat cultures

was controlled at the end of each experiment by plating on TGY agar and transferring all colonies to *E. coli* diagnostic agar plates (ECD-MUG; Biolife).

Specific growth rates in batch cultures were calculated by linear regression of growth curves; in chemostats,  $D$  equals specific growth rate.

**Hydroperoxidase assay and  $\beta$ -galactosidase activity.** The two *E. coli* hydroperoxidases (catalases) were used as 'reporter genes' for RpoS-dependent transcription because both hydroperoxidase I (*katG* gene product, cytoplasmic membrane associated) and hydroperoxidase II (*katE* gene product, present in the cytosol) were shown to be regulated by  $\sigma^s$  (Ivanova *et al.*, 1994), with hydroperoxidase II expression being entirely dependent on RpoS (Visick & Clarke, 1997). Hydroperoxidase I (HPI) and hydroperoxidase II (HPII) specific activities were measured as described by Visick & Clarke (1997). Briefly, *E. coli* cells were sampled directly onto ice. *De novo* protein synthesis was stopped by adding 25  $\mu$ g chloramphenicol  $\text{ml}^{-1}$ . After centrifugation at 6000  $g$ , the cell pellet was resuspended in 200  $\mu$ l lysis buffer and cells were lysed by sonication (Sonifier 450; Branson; power setting of 3), while being cooled on ice. The extract was centrifuged for 10 min at 6000  $g$  to remove cell debris.  $\text{H}_2\text{O}_2$ -degrading activity was determined by diluting 50  $\mu$ l of the supernatant (crude extract) in 2 ml of 50 mM sodium phosphate buffer (pH 7.0, 37°C), adding 4  $\mu$ l  $\text{H}_2\text{O}_2$  (37%) and then following the decrease in absorbance at 240 nm over time. The protein concentration in the crude extract was determined with a commercial bichinoic acid assay (Bio-Rad). The specific activity of hydroperoxidase was calculated following Visick & Clarke (1997).

HPI and HPII were differentiated by heating an aliquot of the crude extract for 15 min at 55°C. Visick & Clarke (1997) demonstrated that HPI is heat labile at 55°C, whereas HPII is stable. We could verify the strict RpoS-dependency of HPII expression by comparing an *rpoS*<sup>0</sup> allele of our strain with the wild-type (Table 1, Fig. 4).

$\beta$ -Galactosidase activity was measured, as described elsewhere (Miller, 1972), by using SDS- and chloroform-treated cells from chemostat cultures in appropriate amounts.

**Detection of *rpoS* mutants.** RpoS null and partial mutants were distinguished from the wild-type by iodine staining of colonies grown on TGY agar plates and incubated overnight at 4°C

(Notley-McRobb *et al.*, 2002). Dark-brown colonies were counted as *rpoS* positive, whereas pale-brown colonies were assumed to be *rpoS*-attenuated or null mutants.

#### Protein extraction and Western blotting analysis of RpoS.

Cells for immunoblot analysis were sampled directly onto ice and centrifuged at 4°C. After carefully removing the supernatant, the pellet was immediately frozen at -20°C. The remaining water was removed by freeze-drying. Before loading onto a 10% acrylamide gel, the pellet was resuspended in SDS-PAGE sample buffer and boiled for 5 min. The amount of sample buffer added was normalized to the optical density of the initial culture fluid/ice mixture. Total band intensity on Coomassie-stained SDS-PAGE control gels showed that this procedure led to approximately the same protein concentrations in samples. Protein extraction, by heating samples supplemented with 0.1 M KOH to 80°C for 40 min, also led to similar protein concentrations after normalization of optical density (as determined with a bichinoic acid assay). To verify results obtained with the above sampling method, protein was also extracted by direct precipitation with 10% TCA (Lange & Hengge-Aronis, 1994). Because of loss of protein during washing steps, loading volumes had to be adjusted by normalizing to total band intensity on Coomassie-stained gels. Relative band intensities were quantified with the publicly available software ImageJ (<http://rsb.info.nih.gov/ij/download.html>).

Following SDS-PAGE, proteins were blotted overnight (4°C, 30 V) onto nitrocellulose membranes. The transfer buffer contained 25 mM Tris, 192 mM glycine and 20% methanol. After blotting, the membrane was blocked for 1 h in TBSTM (20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.05% v/v, Tween 20, 5% w/v, skimmed milk powder), washed for 10 min with TBSTM and incubated for 2 h with mouse monoclonal antibodies against RpoS or RpoD (Neoclone, Madison, WI, USA) which were both diluted in TBSTM. Following two further TBSTM washing steps, membranes were hybridized for 1 h with goat anti-mouse antibodies coupled to alkaline phosphatase (Bio-Rad). RpoS bands were visualized by reaction with BCIP/NBT (Sigma-Aldrich) and identified by comparison to a *rpoS*-negative control strain (Fig. 2).

**Quantification of glucose and acetate.** Glucose in concentrations above 10  $\text{mg l}^{-1}$  was determined with a commercially available glucose oxidase assay (Boehringer Mannheim). Concentrations

**Table 1.** Effect of final cell density and medium composition on HPI and HPII specific activities in overnight stationary phase batch cultures

HPI and HPII specific activities are given in  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} (\text{mg total protein})^{-1}$  and are the mean of three replicate experiments. Values in parentheses show SD.

Strain and medium	Final OD <sub>546</sub>	HPI	HPII
<b><i>E. coli</i> MG1655 wt</b>			
LB*	5.9 ( $\pm 0.5$ )	43.2 ( $\pm 1.5$ )	87.4 ( $\pm 6.0$ )
LB 1:100†	0.063 ( $\pm 0.008$ )	33.3 ( $\pm 4.3$ )	79.8 ( $\pm 8.6$ )
LB 1:100, without NaCl‡	0.050 ( $\pm 0.005$ )	48.0 ( $\pm 4.3$ )	68.1 ( $\pm 13.6$ )
Glucose mineral medium§	2.7 ( $\pm 0.1$ )	21.2 ( $\pm 1.2$ )	35.0 ( $\pm 1.2$ )
<b>MG1655 <i>rpoS13::Tn10</i></b>			
LB	4.9 ( $\pm 0.2$ )	85.1 ( $\pm 2.3$ )	1.0 ( $\pm 0.6$ )

\*pH<sub>end</sub> = 8.2.

†100 mg tryptone, 50 mg yeast extract and 10 g NaCl per litre. pH<sub>end</sub> = 6.8.

‡100 mg tryptone, 50 mg yeast extract. pH<sub>end</sub> = 7.26.

§Medium C<sub>lim</sub>Ba containing 4 g glucose  $\text{l}^{-1}$ .

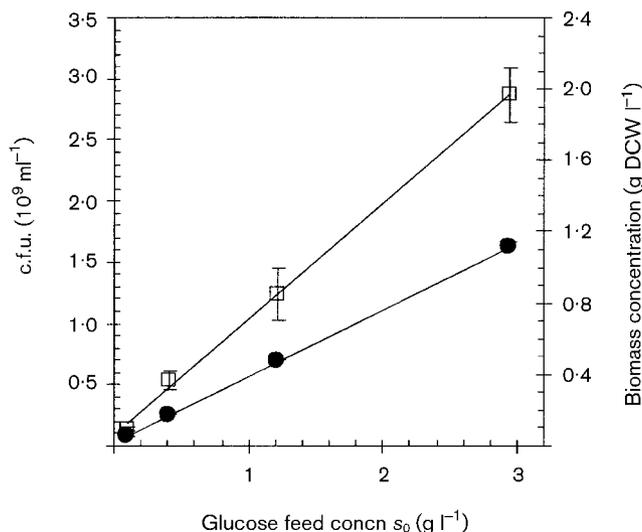
below  $10 \text{ mg l}^{-1}$  were analysed by ion chromatography using a Carbpac PA10 column (Dionex). The eluent was  $0.2 \text{ M NaOH}$ , and the analyte was measured by pulsed amperometric detection (detection limit  $10 \mu\text{g l}^{-1}$ , linear range  $10\text{--}1000 \mu\text{g l}^{-1}$ ). Acetate was analysed by ion chromatography using an IonPac ICE AS-6 column (Dionex) and chemical suppression with  $5 \text{ mM}$  tributyl ammonium hydroxide. Heptafluorobutyric acid at a concentration of  $0.4 \text{ mM}$  was used as eluent and the analyte was measured by conductivity detection (detection limit  $3 \text{ mg l}^{-1}$ , linear range  $3\text{--}200 \text{ mg l}^{-1}$ ). Calibration curves were obtained by measuring analyte solutions of known concentration.

## RESULTS

### RpoS is not regulated by cell density

Cell density was linearly dependent on glucose feed concentration ( $s_0$ ) up to  $3 \text{ g l}^{-1}$  in chemostat cultures of strain MG1655 fed with carbon-limited medium  $C_{\text{lim}}\text{Ch}$  (Fig. 1). The intracellular concentration of RpoS at a dilution rate of  $0.3 \text{ h}^{-1}$  was similar at all cell densities from  $1.4 \times 10^8\text{--}2.9 \times 10^9 \text{ c.f.u. ml}^{-1}$  (Fig. 2a). Both sampling procedures, TCA precipitation, and direct protein extraction in SDS-PAGE sample buffer, led to similar band intensities (Fig. 2a). This justifies the use of the simpler direct extraction method for further immunoblot analyses.

Hydroperoxidase assays, which were used as a measure of RpoS-dependent gene expression, confirmed the immunoblot results. Both HPI and HPII specific activities decreased by 40%, rather than increased, from low to high cell density (Fig. 3a). HPII accounted for 50–60% of total hydroperoxidase activity at all cell densities tested (Fig. 3a). In additional independent chemostat runs, whole-cell hydroperoxidase activity (representing HPI and HPII combined



**Fig. 1.** Linearity of cell density as c.f.u. ( $\square$ ) and biomass concentration ( $\bullet$ ) in relation to glucose feed concentration in carbon-limited chemostat cultures of *E. coli* MG1655 ( $D=0.3 \text{ h}^{-1}$ ). Error bars show SD of five replicate measurements.

activity) declined by about 50% from low to high cell density (data not shown).

Independence of RpoS expression from cell density was also observed in batch culture experiments. Cultivation into stationary phase in 100-fold diluted LB resulted in about the same intracellular RpoS levels as cultivation in full-strength LB (Fig. 2d). HPI and HPII specific activities in overnight LB cultures were also unaffected by a 100-fold difference in final cell density (Table 1).

### Type of limitation does not influence RpoS levels

Due to high external glucose concentrations, cAMP levels in nitrogen- (i.e. non-carbon-) limited growing cells are very low compared to those in carbon-limited growing cells (Notley-McRobb *et al.*, 1997). Furthermore, acetate concentrations in non-carbon-limited chemostat cultures are high, due to overflow metabolism (Neijssel *et al.*, 1996; Table 2). Although both cAMP and acetate have been reported to influence RpoS levels, we found no difference in intracellular RpoS concentrations in nitrogen- and iron-limited chemostats, compared to carbon-limited chemostats operated at the same dilution rate (Fig. 2b).

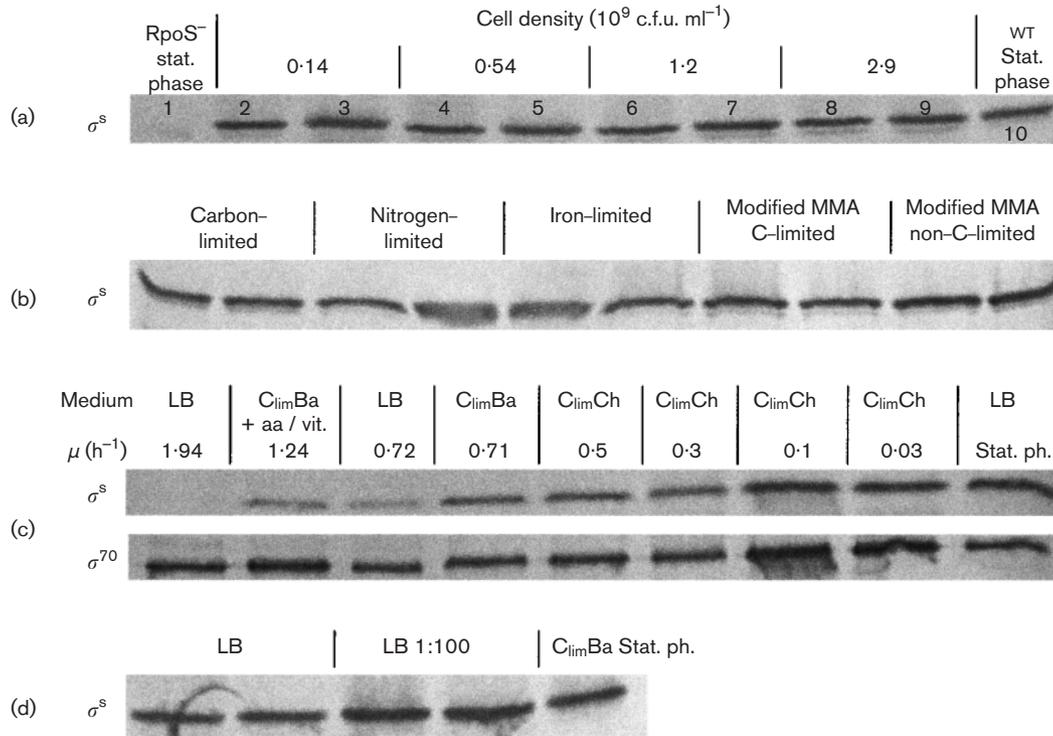
In addition, HPI and HPII specific activities were virtually the same in nitrogen- and carbon-limited chemostat cultures of similar cell density (Fig. 3; for cell density of the nitrogen-limited chemostat see Table 2). However, HPII specific activity was strongly reduced under iron-limited growth conditions and HPI activity was absent (Fig. 3b).

A direct test of the effects of cAMP and acetate on RpoS levels in chemostat cultures also indicated that differences in the nature of the limiting nutrient have no effect on RpoS expression. Neither a pulse of  $0.45 \text{ mM}$  cAMP into a nitrogen-limited chemostat culture, nor the addition of  $0.4 \text{ g l}^{-1}$  ( $24 \text{ mM}$ ) acetate to the highest cell density carbon-limited chemostat culture had an effect on RpoS immunoblot band intensities (data not shown).

### RpoS levels and hydroperoxidase specific activity are strongly affected by specific growth rate

RpoS band-intensity on immunoblots inversely correlated with specific growth rate in exponential-phase batch cultures and steady-state chemostat cultures, whereas the level of housekeeping sigma factor  $\sigma^{70}$  (RpoD) was not much affected (Fig. 2c). Intracellular RpoS concentration was also influenced by amino acid availability, with a higher level in amino-acid-free mineral medium cultures compared to an LB chemostat culture growing at the same specific growth rate (Fig. 2c). All samples were taken at low cell density (i.e. below an  $\text{OD}_{546}$  of  $0.25$ , corresponding to  $0.1 \text{ g DCW l}^{-1}$ ), which demonstrates that regulation by specific growth rate proceeds independently of cell density.

The specific activities of HPI and HPII were also strongly



**Fig. 2.** Effect of cell density, type of limitation and specific growth rate on intracellular  $\sigma^S$  and  $\sigma^{70}$  concentrations in *E. coli* MG1655, as determined by immunoblot analysis. (a)  $\sigma^S$  levels at different steady-state cell densities in glucose-limited chemostat cultures ( $D=0.3$  h<sup>-1</sup>). Positive and negative control samples were taken from stationary-phase cultures with glucose-limited mineral medium (C<sub>lim</sub>Ba). Lanes 1, 2, 4, 6, 8, 10, direct protein extraction in SDS-PAGE sample buffer; lanes 3, 5, 7, 9, TCA precipitation. (b) Two samples taken at different time points from carbon- and non-carbon-limited chemostat cultures ( $D=0.3$  h<sup>-1</sup>), direct protein extraction. (c)  $\sigma^S$  and  $\sigma^{70}$  levels at different specific growth rates in exponential-phase batch and chemostat cultures, direct protein extraction. For further details see legend to Fig. 4. (d)  $\sigma^S$  expression in two individual stationary-phase cultures, each in LB and 100-fold diluted LB. Parameters at time of sampling: optical density >90% of final value; specific growth rate <0.1 h<sup>-1</sup>. WT, Wild-type.

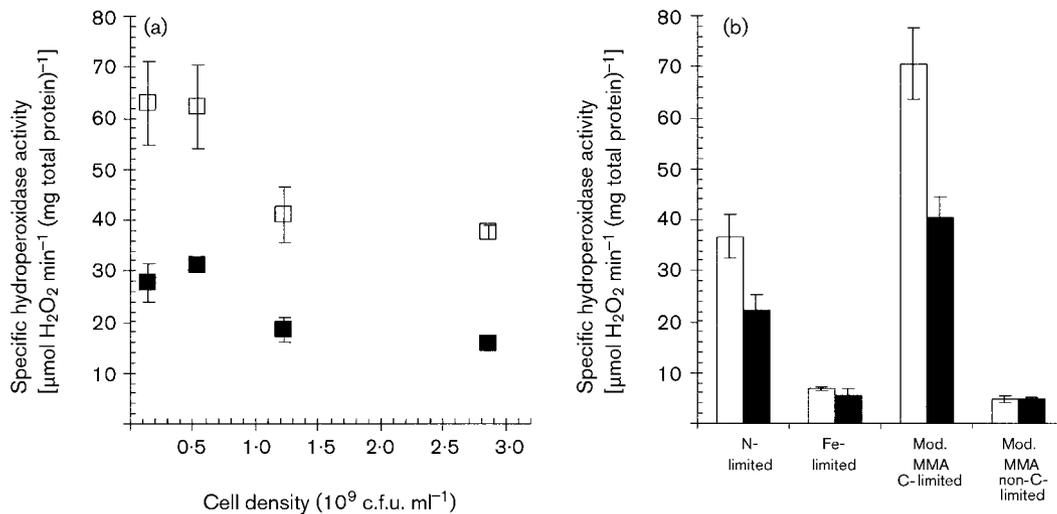
regulated by specific growth rate (Fig. 4). Expression ranged from almost complete repression at  $\mu=1.94$  h<sup>-1</sup> to maximum specific activities of 54 (HPI) and 81 (HPII)  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$  at the lowest specific growth rate tested (0.03 h<sup>-1</sup>). Interestingly, HPII specific activity was much higher in amino-acid-free mineral medium culture than in an amino-acid-containing LB culture, in spite of similar specific growth rates (Fig. 4), whereas HPI specific activity was much higher in the LB culture, leading to approximately similar HPI and HPII combined activities (Fig. 4). Cultivation to stationary phase in mineral medium resulted in RpoS and hydroperoxidase levels similar to those of chemostat cultures with a dilution rate of 0.3 h<sup>-1</sup>, whereas stationary phase in LB culture induced twofold higher HPI and HPII levels, in the same range as those of chemostat cultures with a dilution rate of 0.03 h<sup>-1</sup> (Figs 2 and 4, Table 1). This difference is not due to the presence of sodium chloride in LB because overnight LB cultures with and without 10 g sodium chloride l<sup>-1</sup> had similar hydroperoxidase specific activities (Table 1).

In spite of loss of RpoS function, HPI specific activity was strongly elevated in overnight batch and chemostat cultures of the *rpoS*<sup>0</sup> strain, leading to total hydroperoxidase specific activities in the same range as those of the wild-type strain (Fig. 4, Table 1).

### High-cell-density MMA chemostat cultures become non-carbon limited

In an earlier chemostat study in which cell-density effects on *E. coli* were reported, MMA was used as growth medium (Liu *et al.*, 2000). However, MMA lacks trace elements (Miller, 1972). Thus, we tested whether the observed effects might be due to a hidden secondary limitation in high glucose-feed chemostat cultures.

Nitrogen- and iron-limited (i.e. non-carbon-limited) chemostat cultures of *E. coli* MG1655 were characterized by a 40–50% lower growth-yield compared to glucose-limited cultures with medium C<sub>lim</sub>Ch (Table 2). Furthermore, 20% of the glucose carbon consumed was transformed to acetate



**Fig. 3.** Effect of (a) cell density and (b) type of limitation on specific hydroperoxidase activity in steady-state chemostat cultures ( $D=0.3 \text{ h}^{-1}$ ). □ and white bars, combined HPI and HPII specific activities; ■ and black bars, HPII specific activity. MMA chemostats run with low glucose feed ( $0.1 \text{ g l}^{-1}$ ) were carbon-limited, whereas high-glucose-feed MMA cultures ( $5.75 \text{ g l}^{-1}$ ) were not. Error bars show SD of three replicate measurements. Mod. MMA, modified MMA.

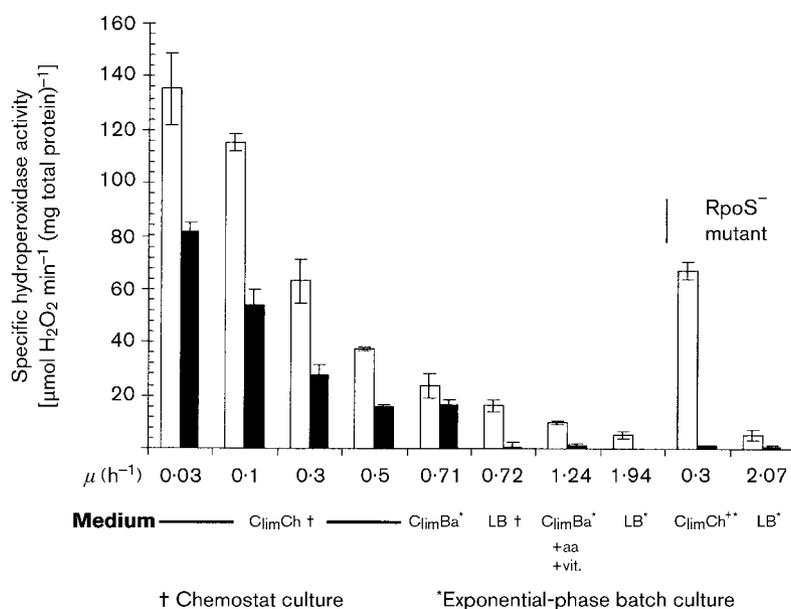
(calculated from values in Table 2). This is in agreement with data reported for nitrogen-, phosphorus- and potassium-limited chemostat cultures of *E. coli* strain PC-1000 (Neijssel *et al.*, 1996). Judged from growth yield, steady-state glucose concentration and acetate excretion, MMA and modified MMA sustained purely carbon-limited growth in chemostats fed with  $0.1 \text{ g glucose l}^{-1}$  (Table 2), whereas high glucose feed resulted in non-carbon limitation

(Table 2). In spite of  $6 \text{ g l}^{-1}$  glucose feed, the maximum  $\text{OD}_{546}$  obtainable with MMA in chemostat culture was  $1.7$  (Table 2), which corresponds to  $0.5 \text{ g DCW l}^{-1}$ . Addition of  $77 \text{ mg l}^{-1} \text{ FeCl}_3 \cdot 6\text{H}_2\text{O}$  to a high-glucose-feed MMA chemostat culture of strain BW2952 led to an increase in  $\text{OD}_{546}$  from  $1.7$  to  $2.9$ . These findings suggest that MMA is unsuitable for growing bacterial cells to densities above  $0.5 \text{ g DCW l}^{-1}$  because the cultures become iron-limited.

**Table 2.** Culture characteristics of chemostats operated to study the effect of cell density and the type of limitation on RpoS

Yield represents the biomass dry weight concentration divided by the difference of glucose feed and steady-state concentrations. Values shown in the table are the mean of a minimum of three replicate samples, except for yield values, which are the mean of a minimum of five replicate samples. Values in parentheses show SD. ND, Not determined.  $D$  was  $0.3 \text{ h}^{-1}$  throughout.

Strain and medium	Glucose feed concn ( $\text{g l}^{-1}$ )	$\text{OD}_{546}$	Yield ( $\text{g dry wt g Glc}^{-1}$ )	Steady-state glucose concn ( $\text{g l}^{-1}$ )	Steady-state acetate concn ( $\text{g l}^{-1}$ )
<b><i>E. coli</i> MG1655</b>					
$C_{\text{lim}}\text{Ch}$	$0.10 (\pm 0.0005)$	$0.11 (\pm 0.004)$	$0.46 (\pm 0.006)$	$0.00037 (\pm 0.0001)$	$<0.003$
$C_{\text{lim}}\text{Ch}$	$0.41 (\pm 0.002)$	$0.47 (\pm 0.005)$	$0.42 (\pm 0.007)$	$<0.0005$	$<0.003$
$C_{\text{lim}}\text{Ch}$	$1.22 (\pm 0.08)$	$1.40 (\pm 0.02)$	$0.39 (\pm 0.004)$	$<0.0005$	$<0.003$
$C_{\text{lim}}\text{Ch}$	$2.94 (\pm 0.02)$	$3.1 (\pm 0.10)$	$0.38 (\pm 0.010)$	$<0.0005$	$<0.003$
$N_{\text{lim}}\text{Ch}$	$7.60 (\pm 0.04)$	$2.45 (\pm 0.10)$	$0.22 (\pm 0.006)$	$4.53 (\pm 0.27)$	$0.46 (\pm 0.02)$
$\text{Fe}_{\text{lim}}\text{Ch}$	$7.45 (\pm 0.03)$	$4.47 (\pm 0.07)$	$0.23 (\pm 0.006)$	$1.89 (\pm 0.08)$	$0.91 (\pm 0.07)$
Modified MMA	$0.10 (\pm 0.003)$	$0.11 (\pm 0.003)$	$0.43 (\pm 0.02)$	$0.00049 (\pm 0.00007)$	$<0.003$
Modified MMA	$5.66 (\pm 0.05)$	$1.13 (\pm 0.05)$	$0.18 (\pm 0.015)$	$3.87 (\pm 0.05)$	$0.41 (\pm 0.005)$
<b><i>E. coli</i> BW2952 (MC4100 derivative)</b>					
MMA	$0.09 (\pm 0.003)$	$0.14 (\pm 0.007)$	ND.	$0.00015 (\pm 0.00001)$	$<0.003$
$C_{\text{lim}}\text{Ch}$	$1.40 (\pm 0.01)$	$1.66 (\pm 0.05)$	ND.	$<0.0005$	$<0.003$
MMA	$5.75 (\pm 0.06)$	$1.74 (\pm 0.05)$	ND.	$2.74 (\pm 0.15)$	$0.61 (\pm 0.04)$



**Fig. 4.** Effect of specific growth rate, *rpoS* knockout mutation and medium composition on hydroperoxidase specific activity in cultures of *E. coli* MG1655. White bars, combined HPI and HPII specific activities; black bars, HPII specific activity. All samples from exponential-phase batch cultures were taken at  $\text{OD}_{546} < 0.25$ . Mineral medium chemostats were run with either 0.1 or 0.2  $\text{g l}^{-1}$  glucose feed (low cell density). The LB chemostat culture was fed with 10-fold diluted LB, which resulted in a steady-state  $\text{OD}_{546}$  of 0.14 (26% of maximum optical density in this medium in batch culture). Exponential growth in batch cultures was confirmed by plotting the natural logarithm of  $\text{OD}_{546}$  versus time. Error bars show SD of three independent samples from chemostat cultures or of samples from three independent batch cultures.

Hydroperoxidase specific activity in a high-glucose-feed chemostat culture of MG1655 with modified MMA closely matched that of an iron-limited chemostat (Fig. 3b), but again, RpoS levels were similar in carbon- and non-carbon-limited modified MMA cultures (Fig. 2b).

Medium  $\text{C}_{\text{limCh}}$ , designed to guarantee surplus supply of all essential nutrients, except carbon and energy sources, allowed carbon-limited growth up to 3  $\text{g l}^{-1}$  glucose feed, corresponding to  $\text{OD}_{546}$  of 3.0 and 1.1  $\text{g DCW l}^{-1}$  (Table 2, Fig. 1). At all cell densities tested, residual glucose concentration remained below 0.5  $\text{mg l}^{-1}$ , and acetate was not excreted into the medium in detectable amounts (Table 2).

### Indirect effect of the type of limitation on RpoS levels in *E. coli* BW2952

For the analysis of cell-density effects on MC4100, Liu *et al.* (2000) sampled their continuous cultures after 30 generations. However, recently it was reported that glucose-limited chemostat cultivation strongly selects for loss or attenuation of RpoS function in *E. coli* BW2952, an MC4100 derivative

(Notley-McRobb *et al.*, 2002), so that 96% of the BW2952 population was mutated in *rpoS* after 30 generations (Notley-McRobb *et al.*, 2002). Hence, at the time point of sampling for cell-density effects, low-cell-density, carbon-limited cultures must have already been dominated by *rpoS* mutants. The same authors reported that population takeover by *rpoS* mutants proceeds much more slowly in nitrogen- (i.e. non-carbon-) limited chemostats (Notley-McRobb *et al.*, 2002). Thus, in chemostat experiments with strain MC4100 and MMA as growth medium, a hidden non-carbon limitation at high glucose feed probably slowed down population takeover by *rpoS* mutants and, therefore, mimicked a cell-density effect on RpoS.

The following experimental data support this interpretation. Cells sampled after 30 generations from a low-cell-density, carbon-limited MMA chemostat culture of strain BW2952 exhibited a five-times lower HPII specific activity than cells taken from a high-cell-density, non-carbon-limited MMA chemostat culture (Table 3). Furthermore, a larger proportion of cells had already lost *rpoS* in the low-cell-density chemostat, as judged from dark-brown colonies after iodine

**Table 3.** Effect of glucose feed concentration on HPII specific activity, loss of *rpoS* and *malG-lacZ*  $\beta$ -galactosidase activity in chemostat cultures of *E. coli* BW2952

MMA was used as growth medium and  $D$  was 0.3  $\text{h}^{-1}$ . Samples were taken 30 generations after inoculation. HPII specific activities are given in  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} (\text{mg total protein})^{-1}$  and are the mean of three replicate samples. Values in parentheses show SD.

Glucose feed concn ( $\text{g l}^{-1}$ )	Limitation	HPII	Proportion of <i>RpoS</i> <sup>+</sup> cells (%)	<i>malG-lacZ</i> $\beta$ -Galactosidase activity (Miller units)
0.1	Carbon	7.7 ( $\pm 5.5$ )	2.4 ( $\pm 0.2$ )	549 ( $\pm 9$ )
5.75	Non-carbon	36.7 ( $\pm 3.9$ )	6.0 ( $\pm 3.7$ )	3.2 ( $\pm 0.6$ )

staining and from *malG-lacZ* activity (Table 3), which was in the same range as values reported for *rpoS* mutants of BW2952 (Notley-McRobb *et al.*, 2002).

One could argue that loss of *rpoS* also distorted results obtained in our chemostat experiments with strain MG1655. However, although MG1655 attenuates RpoS expression in the long term, i.e. between 50 and 215 generations (unpublished results), no *rpoS* mutants were detected in glucose-limited chemostats after 30 generations, which was the maximum experimental time-span investigated in this study. Differences in the stability of RpoS phenotype between MG1655 and MC4100 are probably related to the significant differences in genetic background (see Methods and Peters *et al.*, 2003).

## DISCUSSION

### RpoS expression in *E. coli* is not controlled by quorum sensing

In the last few years, quorum sensing has increasingly been used to explain complex microbial phenomena. Quorum sensing *sensu stricto* means that bacterial cells produce membrane-diffusible ('quorum sensing') signal molecules which accumulate in the medium in parallel with cell density and, upon reaching a certain threshold concentration, induce quorum-sensing-dependent genes. Not surprisingly, the *rpoS*-dependent general stress response has been proposed to be one of the regulatory networks influenced by quorum sensing (Lange & Hengge-Aronis, 1994; Huisman & Kolter, 1994; Hengge-Aronis, 1996, 2000) because its induction is inevitably accompanied by an increase in cell density in standard batch-culture experiments (Lange & Hengge-Aronis, 1994; Jishage *et al.*, 1996).

However, the evidence from chemostat and batch experiments presented in this paper strongly argues against any type of quorum-sensing regulation of RpoS in *E. coli*. In neither chemostat nor batch cultures did increasing cell density lead to higher RpoS levels or elevated RpoS-dependent HPII expression. The cell densities tested in our study cover the range in which quorum-sensing-dependent induction is usually reported to take place (Latifi *et al.*, 1996; Surette & Bassler, 1998). Low cell density is supposed to prevent the expression of quorum-sensing-dependent genes, which is clearly not the case for RpoS in our experiments.

Our conclusion that RpoS is not regulated by quorum sensing is backed by the majority of literature data. Several studies showed that supernatant fluid from stationary-phase batch cultures, which should contain quorum-sensing signal molecules, had little or no effect on *rpoS::lacZ* transcriptional and translational fusions (Garcia-Lara *et al.*, 1996; Sitnikov *et al.*, 1996; Hengge-Aronis, 2002). Furthermore, intracellular RpoS concentrations, as well as *rpoS* mRNA levels, did not increase in parallel with cell density in fed-batch culture (Teich *et al.*, 1999; Yoon *et al.*, 2003).

Finally, stationary-phase induction of a *rpoS::lacZ* transcriptional fusion occurred equally well in 100-fold diluted LB and in normal-strength LB (Huisman & Kolter, 1994).

Quorum-sensing in *E. coli* and *Salmonella typhimurium* is said to be mediated by so-called autoinducer 2 (AI-2), the production of which depends on the *luxS* gene product (Surette *et al.*, 1999). However, LuxS plays an important role in central metabolism, i.e. in the recycling of *S*-adenosylmethionine (Winzer *et al.*, 2002). AI-2 may in fact not represent a signal molecule but a metabolite that is released in exponential phase and used as a carbon and energy source in later (carbon-limited) stages of growth (Winzer *et al.*, 2002). Even if one accepts the role of AI-2 as a signal molecule, it seems not to be important for *rpoS* regulation, because *rpoS* transcription was not induced significantly in a *luxS* mutant by the addition of AI-2-containing culture supernatant (DeLisa *et al.*, 2001). Likewise, a transcriptome analysis with *E. coli* EHEC O157:H7 showed that *rpoS* mRNA levels are not affected by a *luxS* knockout mutation (Sperandio *et al.*, 2001).

There is one study which reported, at first sight, convincing evidence for cell-density effects on gene expression in *E. coli*, including an inducing effect on RpoS (Liu *et al.*, 2000). Results obtained in our chemostat experiments suggest that these effects are an artefact caused by a hidden, secondary nutritional limitation occurring at high glucose feed in the standard *E. coli* mineral medium MMA (discussed below). The observed effect of cell density on RpoS levels in the strain employed by Liu *et al.* (2000) is probably caused by differential loss of RpoS function in low- and high-cell-density MMA chemostats (see also Results, indirect effect of the type of limitation on RpoS levels in *E. coli* BW2952).

From an ecological point of view, dependence on signals from other cells for starvation survival seems to be quite risky. An 'egoistic' approach, where each bacterial cell reacts on its own, efficiently and quickly to stresses and changes in its environment, might ensure a better survival of the species. The fact that *E. coli* is usually present in its primary habitat at rather low cell densities of  $10^6$  per g colon content (Smith, 1965) also speaks against intraspecies quorum sensing being important for this bacterium.

We speculate that a similar set of experiments to those described in this study might reveal that RpoS expression proceeds independently of cell density in other microorganisms as well, and particularly in *P. aeruginosa*, considering the conflicting data published for that organism (Latifi *et al.*, 1996; van Delden *et al.*, 2001).

### The type of limitation is irrelevant to RpoS expression

In chemostats operated at  $D=0.3 \text{ h}^{-1}$ , RpoS was expressed at approximately the same intracellular concentrations, regardless of whether the cultures were carbon-, nitrogen- or iron-limited (Fig. 2b). This is in agreement with batch

experiments showing that glucose and phosphate limitation lead to a similar increase in RpoS during growth arrest (Gentry *et al.*, 1993). Strictly *rpoS*-dependent *stiA* and *stiC* genes were also induced to about the same level during carbon, nitrogen and phosphate starvation (O'Neal *et al.*, 1994). Higher RpoS levels in nitrogen-limited compared to glucose-limited chemostat cultures of strain MC4100 (Liu & Ferenci, 2001) are most likely due to a rapid loss of *rpoS* function under glucose limitation, in contrast to a slow loss under nitrogen limitation (Notley-McRobb *et al.*, 2002), and thus are not valid as counter-evidence.

### Specific growth rate plays a prominent role in the regulation of the general stress response

Data presented in this study indicate a central role for specific growth rate in RpoS regulation in the absence of stresses other than nutritional limitation. Both RpoS itself and RpoS-dependent hydroperoxidase expression exhibited a strong negative correlation to specific growth rate, irrespective of cell density. This suggests that the increase in intracellular RpoS concentrations in LB batch cultures (Gentry *et al.*, 1993; Lange & Hengge-Aronis, 1994; Jishage *et al.*, 1996) is mainly triggered by a gradual decline in specific growth rate. A strong negative correlation to specific growth rate was also shown for RpoS-dependent *osmY-lacZ* activity and trehalase expression in glucose-limited chemostats (Notley & Ferenci, 1996). However, the tendency of MC4100 derivative strains quickly to lose *rpoS* function in chemostats (Notley-McRobb *et al.*, 2002), and the *relA1* mutation in MC4100, which might affect RpoS expression, add some uncertainty to these results. Reduction in specific growth rate from 0.8 to 0.1 h<sup>-1</sup> in chemostat cultures of strain RB791 (*F<sup>-</sup> INrrnD-rrnE1 λ<sup>-</sup> lac<sup>F</sup>L<sub>8</sub>*) led to a twofold increase in steady-state RpoS levels (Teich *et al.*, 1999), which is in the same range as a 2.4-fold increase from  $\mu=0.7$  h<sup>-1</sup> to  $\mu=0.1$  h<sup>-1</sup> observed in our study (calculated from background-corrected band intensities in Fig. 2c).

A stronger induction of the general stress response in stationary-phase LB cultures compared to mineral glucose medium (observed by Lange & Hengge-Aronis, 1994 and confirmed in this study) might be due to the extended period of very slow growth when cultures approach stationary phase in LB. Contrary to this, cultivation in mineral glucose medium is characterized by a sudden drop in specific growth rate to zero (Lange & Hengge-Aronis, 1994) and, presumably, cells entering stationary phase in mineral medium do not have enough time and resources to induce a strong general stress response. The conditions that bacteria encounter when leaving the colon probably rather resemble those found in late stationary-phase LB, with a period of slow growth on hard-to-use substrates left in the faeces.

### Which signals link specific growth rate and stress defence?

A possible intracellular signal that links RpoS and specific growth rate is (p)ppGpp, because RpoS is positively

regulated by this factor (Gentry *et al.*, 1993; Lange *et al.*, 1995) and (p)ppGpp levels rise in response to amino-acid deprivation (Cashel *et al.*, 1996), carbon starvation (Cashel *et al.*, 1996) and reduction in specific growth rate (Teich *et al.*, 1999). Not only does (p)ppGpp induce RpoS itself, but it also improves the ability of  $\sigma^S$  to compete for core RNA polymerase (Jishage *et al.*, 2002) and thus enhances *rpoS*-dependent transcription. Therefore, the strong effect of the presence or absence of amino acids in the medium on strictly RpoS-dependent HPII specific activity (Fig. 4) might be due to different intracellular levels of (p)ppGpp.

Although HPI also correlated strongly with specific growth rate (Fig. 4, difference between white and black bars), its expression was not dependent on functional RpoS in a glucose-limited chemostat (Fig. 4). Similarly, HPI was expressed to quite high levels in stationary-phase LB cultures of our *rpoS* knockout strain (Table 1), which confirms results reported by Visick & Clarke (1997). Interestingly, the pattern of hydroperoxidase expression in a wild-type chemostat culture with LB complex medium was quite similar to *rpoS* mutant cultures, with relatively high HPI specific activities and a virtual absence of HPII (Fig. 4). Due to the presence of amino acids, LB chemostat cultures are likely to be low in intracellular (p)ppGpp, which in turn could suppress RpoS itself and RpoS-dependent transcription initiation. Thus, it seems that HPI can take over as H<sub>2</sub>O<sub>2</sub>-degrading enzyme under low (p)ppGpp conditions or when functional RpoS is absent, which implies that additional mechanisms apart from (p)ppGpp-RpoS link specific growth rate and stress defence in *E. coli*. Possible mechanisms include changes in DNA topology (Drlica, 1992), and a system that senses oxidative damage resulting from the cell's own respiratory activity, which increasingly becomes a problem with decreasing specific growth rate (Nyström, 1998).

### The importance of medium design for high-cell-density cultivation

Bacterial growth is dependent on adequate supply of all essential nutrients that cannot be synthesized by the cells themselves. MMA (Miller, 1972) and medium M9 are the two most-used mineral media for the cultivation of *E. coli*. Despite their widespread application, both media lack trace elements, particularly iron. Iron is necessary in considerable amounts for aerobic growth, so that the term 'trace element' is actually not fully appropriate (Egli, 2000). Not surprisingly, the highest cell density obtainable with MMA in chemostat culture was considerably lower than cell densities reached in trace-element-containing medium C<sub>lim</sub>Ch (Table 2). In spite of the use of stainless steel bioreactors, iron seemed to be the limiting nutrient in MMA. A theoretical analysis of medium MMA, based on elemental growth yields (Egli, 2000), excludes a possible N-, P-, K- or S-limitation and leaves iron as the most probable growth-limiting nutrient.

Hence, a shift from glucose limitation at low cell density to

dual glucose/iron limitation (Egli, 1997) at high cell density in experiments performed with medium MMA (Liu *et al.*, 2000) is very likely. Such an interpretation fits well with the increase in intracellular glucose, UDP-glucose and UDP-N-acetylglucosamine concentration with cell density (Liu *et al.*, 2000), indicating improved glucose availability and thus a shift to non-carbon limitation. Furthermore, the change in porin expression from predominantly OmpF to OmpC (Liu *et al.*, 2000) also argues for high-cell-density MMA cultures becoming non-carbon limited, because the same authors reported earlier that OmpF is much more strongly expressed than OmpC in glucose-limited chemostats (Liu & Ferenci, 1998), whereas OmpC dominated over OmpF in nitrogen-limited chemostats (Liu & Ferenci, 1998).

A thorough investigation of limitations affecting growth in MMA and M9 media requires further research.

### Are hydroperoxidases in *E. coli* regulated by iron?

Hydroperoxidase specific activity was strongly decreased in iron-limited chemostats (Fig. 2b). A negative effect of iron starvation on hydroperoxidase activity was also observed for the opportunistic pathogen *P. aeruginosa* (Frederick *et al.*, 2001). The lowered hydroperoxidase specific activity could reflect either an iron deficiency-mediated repression of hydroperoxidase genes or a shortage of functional haem groups resulting in many non-functional hydroperoxidase proteins. It has been observed that iron-free cofactors are excreted into the medium under iron limitation (Townsend & Neilands, 1956), and that total haem iron is significantly decreased in iron-limited growing yeast cells (Light, 1972). Furthermore, expression of the iron regulator Fur is linked to the oxidative stress response through OxyR (Zheng *et al.*, 1999). However, although the iron-dependent superoxide dismutase SodB is regulated by Fur, this is not the case for KatG (HPI) and KatE (HPII) (Hantke, 2001).

The effect of iron deficiency on hydroperoxidase specific activity offers an explanation for the 40% decrease in HPI and HPII activity seen at high cell density (Fig. 3a). Although culture parameters do not indicate iron or dual carbon/iron limitation during growth in medium C<sub>lim</sub>Ch (Table 2), high cell density nevertheless might lead to difficulties in iron acquisition because of a lowered Fe<sup>3+</sup> : [Fe<sup>3+</sup> EDTA] ratio compared to that at low cell density. A reduced supply of iron then would cause a reduction in hydroperoxidase specific activity.

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