Proton-motive force during growth of *Escherichia coli* in the recycling fermentor

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The proton-motive force (Δp) has been determined during growth of *Escherichia coli* in a recycling fermentor and in a chemostat. The recycling fermentor has proven to be an easy and suitable technique for measuring Δp in growing cells, especially at low specific growth rates. Δp was determined by measuring the transmembrane gradient of protons, ΔpH, from the accumulation of benzoate anions, and the electrical gradient, $\Delta\psi$, from the accumulation of the lipophilic cation tetraphenylphosphonium (TPP^+). The accumulation of lactose was also used to calculate Δp in two lactose operon constitutive, β-galactosidase negative relaxed mutants. In the recycling fermentor the calculated Δp values were independent of the specific growth rate and of the growth phase with both relaxed mutants. Δp values measured in the chemostat at different specific growth rates yielded the same result. Results with a wild-type *E. coli* that expresses the stringent response at specific growth rates lower than 0.01 h^{-1}, corresponding to generation times greater than 70 h, showed that Δp varies inversely with specific growth rate. These results indicate that a relationship exists between Δp and the expression of the stringent response.

Protonmotive force *Recycling fermentor* *Chemostat* *Stringent response* *ppGpp*

1. INTRODUCTION

The electrochemical proton gradient ($\Delta\mu H^+$), which gives rise to the proton-motive force (Δp), is one of the main driving forces for energy-requiring processes. It couples metabolic energy to transport of nutrients and to ATP synthesis [1,2]. Δp also may be involved in the regulation of metabolic processes, such as nitrogen fixation [3], DNA uptake during transformation [4], and ppGpp breakdown [5]. The involvement of the Δp in ppGpp breakdown has been of special interest for recent studies on the stringent response of cells cultured in the recycling fermentor [6–10].

Considerable information has been obtained on the magnitude of the proton-motive force in non-growing bacterial cells and cells growing in a batch culture [11–16], recently reviewed by Kashket [17]. However, only one report has appeared thus far describing the relation between specific growth rate and Δp measured in the chemostat [18]. The authors concluded that the Δp varies inversely with specific growth rate. No data are available about specific growth rates lower than 0.05 h^{-1}, corresponding to generation times (T_d) greater than 14 h, which can be reached only by using the recycling fermentor.

Here we show that the recycling fermentor is suitable for measuring Δp in growing cells, particularly at low specific growth rates.

2. MATERIALS AND METHODS

E. coli strains ML308-225 (lac $i^-z^-y^+a^+$, rel), AM1034 (thi, lac $i^-z^-y^+a^+$, phx, rel) and NF 859 were the experimental organisms. The cells were grown in the basal medium described in [10]. Bacteria were grown in aerobic chemostat cultures at 35°C with a controlled pH of 7 as in [19]. For operation in the recycling mode, the chemostat was

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modified by attaching a 0.2 μm polycarbonate membrane filtration unit that continuously returned the cells to the growth vessel, while removing the filtered medium at a rate equal to the rate of fresh medium input [6]. Biomass was determined by collecting the cells on either 0.2 μm nitrocellulose or polycarbonate (Nuclepore, Pleasanton, CA) membrane filters, drying overnight at 105°C, and weighing; or by total carbon analysis using a Total Carbon Analyzer (type 915A, Beckman Instruments, Fullerton, CA). In the latter case the filtrates were also analyzed to correct for extracellular carbon. The methods were used as parallel measures of biomass.

The proton-motive force was measured by adding radioactive probes to final concentrations as follows: for ΔpH, [^{14}C]benzoate (4 μCi/l; spec. act. 29.4 mCi/mmol); for $\Delta\psi$, [^{14}C]tetraphenylphosphonium bromide (8 μCi/l; spec. act. 31.4 mCi/mmol); for Δp_{lac}, [*D-glucose*-1-^{14}C]lactose (4 μCi/l, spec. act. 57.6 mCi/mmol). 30 and 90 min after addition of the probes samples were removed from the cultures as follows: In the recycling experiments counts of radioactivity in 3 ml samples of the filtrate indicated the extracellular concentration of the probe, and the counts of culture samples yielded, after subtraction of radioactivity found in the filtrate sample, the concentration of intracellular probe. Binding of TPP^+ to cell constituents was estimated after de-energizing the cells with 10% butanol or 50 μM CCCP for 1 h at room temperature, washing twice with 67 mM K phosphate buffer (pH 7), and counting the residual radioactivity. In the chemostat studies culture samples of 5 ml were counted for estimating total probe concentration, and other samples drawn directly from the culture vessel through a disposable membrane filter unit were counted for determining the extracellular probe concentration. Binding of TPP^+, benzoate and lactose to the filters was insignificant. The ΔpH, $\Delta\psi$, Δp and Δp_{lac} values were calculated by using the Nernst equation, as described in [15,20]. The intracellular volume was taken to be 1.63 μl/mg dry wt cells [13].

3. RESULTS AND DISCUSSION

E. coli ML308-225 and AM1034 were grown in an aerobic glucose-limited recycling fermentor. The growth pattern of both strains have been analyzed using a non-linear fitting procedure, in which biomass increase is described according to the Pirt equation [21] as follows

$$dx/dt = (ds/dt - m_s x_t) Y_{xsm} \quad (1)$$

where dx/dt is the increase in biomass (g/h), ds/dt is the substrate provision rate (mol/h), m_s is the maintenance coefficient (mol/g per h), x_t is the total biomass (g) and Y_{xsm} is the maximal growth yield on the substrate, corrected for maintenance (g/mol per h). The growth model and the fitting procedure have been extensively treated by Van Verseveld et al. [22]. The fitting procedure allowed us to describe recycled growth with both *E. coli* strains into two growth phases, which is in accordance with growth patterns found with *Paracoccus denitrificans* and *Bacillus licheniformis* [22]. The fitting procedure yielded values for Y_{xsm} and m_s for growth phase 1 of 96.5 g biomass/mol glucose and 0.32 mmol glucose/g biomass per h, respectively, and for growth phase 2 of 43.6 g biomass/mol glucose and 0.002 mmol glucose/g biomass per h, respectively. During the recycling experiment the cells traversed all specific growth rate (μ) values from 0.14 to 0.01 h^{-1}, corresponding to T_d values of 5–70 h. It was not possible to reach μ values lower than about 0.01 h^{-1}, because of severe foaming of the culture. Growth at μ values lower than 0.01 h^{-1} is characteristic of the stringent response associated with elevated levels of the regulatory nucleotides guanosine 5′-diphosphate-3′-diphosphate (ppGpp) and guanosine 5′-triphosphate-3′-diphosphate (pppGpp) [7–10]. The relaxed status of both *E. coli* strains used here is consistent with the absence of a growth phase showing the stringent response.

E. coli strains ML308-225 and AM1034 were chosen because the absence of β-galactosidase and the constitutivity of the lactose operon offer the opportunity of using the accumulation of lactose as an index of the proton-motive force, although the absence of stringent growth was disadvantageous. The proton-motive force estimated as Δp_{lac} did not change significantly during the recycling experiments. Δp_{lac} was 150–160 mV, independent of growth phase and traversed specific growth rates (0.14–0.01 h^{-1}) (table 1). In strain AM1034 the Δp values were also measured by adding the ΔpH and $\Delta\psi$ components, measured with

Table 1

Proton-motive force of *E. coli* cells grown in the recycling fermentor and in the chemostat

Strain	Δp_{lac} (mV)	59ΔpH (mV)	$\Delta\psi$ (mV)	Δp (mV)
Recycling fermentor				
ML308-225	152 ± 9 (21)	n.d.	n.d.	–
AM1034	157 ± 11 (14)	61 ± 8 (10)	121 ± 5 (10)	182 ± 9 (10)
Chemostat				
NF859, D = 0.05[a]	–	58	88	143
NF859, D = 0.27[a]	–	55	112	170

Δp was measured at pH 7, as described in section 2. Values are means ± SD with the number of observations in parentheses. [a] Mean values of 3 determinations. n.d., not determined. D, dilution rate (l/l per h)

the distribution of benzoate and TPP^+, respectively. The Δp values were somewhat higher than Δp_{lac} values (table 1). Again, no change in Δp was observed during the recycling experiments, confirming the measurements obtained with lactose as the probe.

We also measured Δp in steady-state chemostat cultures of strain AM1034, both as Δp_{lac} and by adding ΔpH and $\Delta\psi$, at dilution rates of 0.25 and 0.05 h^{-1}. The results did not differ significantly from those reported for the recycling experiments. Consequently, no relation between specific growth rate and Δp was seen in both relaxed *E. coli* strains. These results differ from those of Otto et al. [18], who described an inverse relation between specific growth rate and Δp. For that reason we measured Δp in the wild-type *E. coli* NF859, which does not have a constitutive lactose operon and shows a stringent response at low specific growth rates [7]. In the stringent strain the Δp was found to be related to the specific growth rate in the same manner as described by Otto et al. [18] (table 1). ΔpH was not influenced by the growth rate, but the $\Delta\psi$ was significantly higher at a specific growth rate of 0.05 than at 0.27 h^{-1}.

The recycling fermentor has proven to be suitable for measuring Δp in growing cells. The advantages of this technique are that the biomass concentrations are generally high and that extracellular concentrations of probes can be measured in the filtrate. The latter point is important because filtration of culture samples for measuring probe distribution is not necessary, permitting measurements in situ, without disturbing the cells' environment.

The present results confirm the data of Kashket [14], although this is not directly obvious. Kashket measured Δp in growing cells of *E. coli* ML308-225 in batch cultures and in non-growing cells of the same strain. Cells growing in a batch culture have a high specific growth rate because of excess carbon source, and consequently are in growth phase 1. Non-growing cells are in growth phase 2, in which wild-type cells should express the stringent response. No difference in Δp was observed in these two conditions and, indeed, our data confirm this observation. In addition, chemostat experiments showed the independence of Δp of the specific growth rate in strains ML308-225 and AM1034. However, chemostat experiments with wild-type *E. coli* NF859 showed the same inverse relationship between Δp and the specific growth rate as found by Otto et al. [18].

The finding that Δp does not vary with growth rate in both mutant strains could be caused by the *lac* mutation or by the *rel* mutation. We think that the *rel* mutation is the more likely candidate for this behaviour than the *lac* mutation. A consequence of this suggestion is that there exists a relationship between the Δp and the onset of the stringent response. Such a relationship does not imply that the Δp and the stringent response affect each other directly, although the Δp could have a signal function for regulatory mechanisms related to the stringent response (see e.g. [5,23]).

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