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Energetic consequences of multiple K^+ uptake systems in *Escherichia coli*

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The energetics of growth of *Escherichia coli* FRAG 1 under potassium-limited growth conditions and with glucose as sole carbon and energy source were studied in the chemostat and compared with those of a mutant, FRAG 5, defective in the high-affinity potassium uptake system. The steady-state concentration of biomass decreased with increasing growth rate and was the same in both parent and mutant. For each growth rate, the rate of production of ATP was higher in the parent than the mutant strain. Under potassium-limited conditions, FRAG 1 has at least two potassium uptake systems, an inducible high-affinity uptake system and a constitutive low-affinity uptake system (Rhoads, D.B., Waters, F.B. and Epstein, W. (1976) J. Gen. Physiol. 67, 325–341). Apparently, the presence of the high-affinity uptake system in the parent leads to an energy drain. We suggest that this energy drain is due to futile cycling of potassium ions. On the basis of a mosaic non-equilibrium thermodynamic description of bacterial growth, it is concluded that the growth behaviour under potassium limitation corresponds to that expected for a catabolite limitation.

Introduction

Several models have been proposed to describe bacterial growth at different levels of complexity [1–8]. In this paper, the effect of energetic uncoupling on growth behaviour is of special interest and the model developed by Westerhoff and coworkers [9–11] allows a quantitative evaluation of this effect. This so-called mosaic non-equilibrium thermodynamic description considers the metabolism of a bacterium to be composed of three elements: (i) catabolism, in which substrate conversion leads to generation of ATP, (ii) anabolism, in which ATP is used for biosynthesis, and (iii) ATP turnover that is not coupled to either catabolism or anabolism (leak).

A set of equations can be derived in which the flux through each of the pathways is related to the relevant forces [9,11]. Such relations are especially useful for conditions of steady state, e.g., such as can be achieved in chemostat cultures. In the steady state, the relationship between the catabolic flux (J_c) and the anabolic flux (J_a) is described by one of the following equations (slightly simplified from Ref. 9, Eqns. 35 and 36).

$$J_c = \frac{n_p^a}{n_p^c}\left(1+\frac{L_p^1}{1+\left(n_p^a\right)^2 L_a}\right)\left(-J_a\right)+\frac{L_p^1}{n_p^a n_p^c}\left(\Delta G_a-\Delta G_a^{\#}\right) \quad (1)$$

$$J_c = \frac{\frac{n_p^a}{n_p^c}}{\left(1+\frac{L_p^1}{\left(n_p^c\right)^2 L_c}\right)}\left(-J_a\right)+\frac{L_p^1 L_c}{\left(n_p^c\right)^2 L_c+L_p^1}\left(\Delta G_c-\Delta G_c^{\#}\right) \quad (2)$$

The rate of ATP production (J_{ATP}) is related to the catabolic flux: $J_{ATP} = n_p^c J_c$. Each L denotes a

Abbreviation: DNP, 2,4-dinitrophenol.

proportionality constant and each n denotes a stoichiometric coupling constant. The meaning of subscripts or superscripts is: a, anabolism, c, catabolism; p, phosphorylation and l, leak. The first equation can be applied to the condition of catabolite limitation in the chemostat, since in that case the term $\Delta G_a - \Delta G_a^{\#}$ is constant. The second equation is most useful under conditions of anabolite limitation, since then $\Delta G_c - \Delta G_c^{\#}$ is constant [9].

For the purpose of the present paper we are specifically interested in L_p^l, the proportionality constant for the 'ATP leak' reaction. All parameters between brackets denote constants, depending on organism and growth conditions. If conditions are chosen in such a way that L_p^l increases, it may be expected that under catabolite limitation the slope of the line relating catabolism (J_c) to anabolism (J_a) (see Eqn. 1) will increase. Conversely, under anabolite limitation any condition that causes an increase in L_p^l should lead to a decrease in the slope of the line relating catabolism to anabolism (see Eqn. 2).

For many growth conditions the limiting factor may be clear: for instance, ammonia- or sulphate-limited growth of heterotrophic bacteria with glucose as an energy source is clearly anabolite-limited. However, the effect of potassium limitation is more difficult to assess, since under these conditions maintenance of a considerable potassium gradient may constitute an important energy drain. The experimental determination of the effect of increasing 'ATP leak' on the relationship between catabolism and anabolism should allow us to decide whether a particular limitation in the chemostat is related to catabolite or anabolite limitation.

We decided to investigate the effect of increasing 'ATP leak' on the growth behaviour of *Escherichia coli* under potassium-limited conditions in order to determine whether this is a catabolite- or an anabolite-limited condition. Potassium is essential for growth of microorganisms and it serves a number of different functions in the cell [12]. In *E. coli*, potassium can be taken up via a number of systems that differ in their affinity for potassium [13]; there is a constitutive low-affinity system (Trk) and an inducible high-affinity system (Kdp) for potassium uptake. The Trk system consists of a number of elements, Trk A–Trk F, some of which are involved in uptake, while others are related to efflux [13,14]. The precise mechanism of coupling between potassium transport and energy utilisation is not yet known [15–18]. It may be speculated that the high-affinity system (Kdp) is able to maintain a steeper gradient of potassium across the membrane, because the energy input per potassium ion is higher than that for the low-affinity system. If this should be the case, the high-affinity system will pump potassium into the cell to such a level that the low-affinity system will start to operate in reverse. The presence of both high- and low-affinity systems may result in an energy-dissipating futile cycle of potassium (Fig. 1).

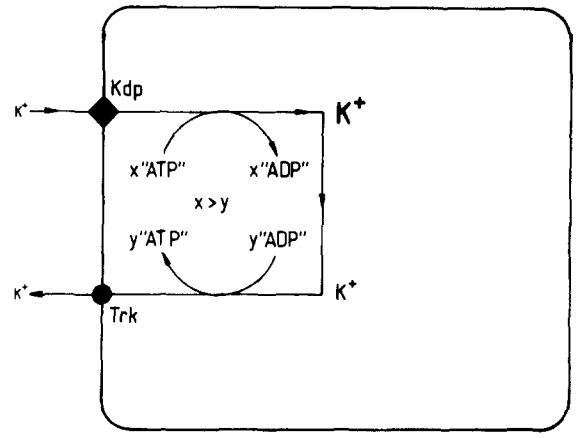

Fig. 1. Schematic representation of the K^+-uptake systems in the membrane of *Escherichia coli*. Two systems with different K^+/'ATP' stoichiometries are postulated. Functioning of both systems together leads to a futile cycle of potassium with a net loss of 'ATP'.

The speculation described above could be tested by comparing *E. coli* cells in which the high-affinity potassium uptake system was either present or absent, by comparing J_{ATP} of *E. coli* wild-type (FRAG 1) with J_{ATP} of a mutant lacking the high-affinity potassium uptake system (FRAG 5) under K^+-limited growth conditions.

In this paper we report experiments in which the parent had a higher 'ATP leak' due to the possibility of futile potassium cycling.

Materials and Methods

Organisms. The following organisms were used in these experiments: *E. coli* FRAG 1 (*thi rha*

lacZ gal) and *E. coli* FRAG 5 (*thi rha lacZ gal KdpABC5*) [12] (kindly donated by W. Epstein, University of Chicago).

Growth conditions. All chemostat experiments were carried out in a 1-liter fermentor (500 series, LH Engineering Co. Ltd., U.K.) with a magnetic stirring device. The culture volume was approx. 700 ml. A simple salts medium was used as specified by Evans et al. [19] with 1 mM K^+ as the limiting factor and 166 mM glucose as sole carbon and energy source. Also, 10 mg thiamine hydrochloride (Sigma) was added per litre of medium. In cultures with 2,4-dinitrophenol, this compound was added up to a final concentration of 0.5 mM DNP. In glucose-limited cultures, 27 mM glucose was added to the medium as the limiting substrate. Media were filter-sterilised, using Millipore filters. The pH value of the cultures was monitored constantly and maintained at 6.9 ± 0.1 by automatic addition of a sterile 4 M NaOH solution. The temperature was set at $35 \pm 1°C$. The cultures were stirred at 1100 rpm and were aerated by flushing 20–30 l air per h through the culture. Foaming was controlled with a foam sensor and by adding a silicone anti-foaming agent (BDH).

In batch culture experiments, *E. coli* FRAG 1 and FRAG 5 were grown on a simple salts medium [19] with 40 μM K^+ as the limiting factor and 22 mM glucose as carbon and energy source. The phosphate concentration was doubled in order to increase the buffering capacity of the medium and 10 mg thiamine hydrochloride per litre of medium was added. Growth was monitored continuously in a Zeiss spectrophotometer as the increase in optical density at 540 nm.

Determination of products Bacterial dry weight was measured by the procedure of Herbert et al. [20]. Products and glucose were separated by HPLC chromatography using a Biorad HPX 87 column and detected with a refractometer (Knauer). Carbon dioxide production was measured with a Servomex PA 404 analyser. Monitoring the flow rate of the effluent gas with a Rotameter, the CO_2 production rate could be calculated.

Determination of potassium. a sample of culture fluid (1 ml) was mounted on top of 0.5 ml silicone oil (3 : 1 mixture of AR200/AR20 Wacker Chemie, München) in an Eppendorf tube. This was centrifuged for 30 s in order to separate biomass and external fluid. The supernatant was used for measurements of external potassium concentrations. The pellet was resuspended in 500 μl of H_2O and afterwards 100 μl perchloric acid (7.5%) was added to determine the internal potassium content. Potassium was measured with a Hitachi atomic absorption spectrophotometer, model 180-80.

Calculation of J_{ATP}. The specific ATP production rate was calculated from the products formed in the chemostat in detectable amounts (CO_2, acetic acid (HAc) and biomass). Succinic acid was formed in negligible amounts. Carbon recovery from glucose was between 90 and 110%. It was assumed that production of pyruvate occurred via glycolysis, accompanied by synthesis of 1 mol ATP + 1 mol NADH per mol of pyruvate. 1 mol biomass, with the empirical formula $C_4H_7O_2N$ [21], was formed from 1 mol pyruvate + 1 mol NADH. The overall equation for biosynthesis from pyruvate and NH_3 is: pyruvate + NH_3 + NADH → biomass. Formation of acetic acid from pyruvate occurred via acetyl-P and was accompanied by synthesis of 1 mol ATP + 1 mol NADH per mol acetic acid and CO_2. Oxidation of pyruvate to CO_2 occurred via the Krebs cycle. The ATP-to-NADH and ATP-to-$FADH_2$ ratios are 2 : 1 and 1 : 1, respectively [22]. On the basis of these metabolic pathways, the ATP production rate, measured in mmol/g per h, can be calculated from the product-formation rates according to the formula:

$$J_{ATP} = \tfrac{26}{6}\left(J_{CO_2} - J_{HAc}\right) + 6\,J_{HAc} + \tfrac{4}{3}\,J_{biomass}$$

Results

E. coli wild type (FRAG 1) and a mutant lacking the high-affinity potassium uptake system (FRAG 5) were cultured aerobically in a chemostat under potassium-limited conditions. As shown in Fig. 2, the bacterial dry weight strongly depended on the growth rate, but there are no gross differences between the two strains. For comparison, in the same figure the effect is given of the uncoupler 2,4-dinitrophenol (DNP) on the dry weight of the mutant under the same conditions.

Under potassium-limited conditions glucose is

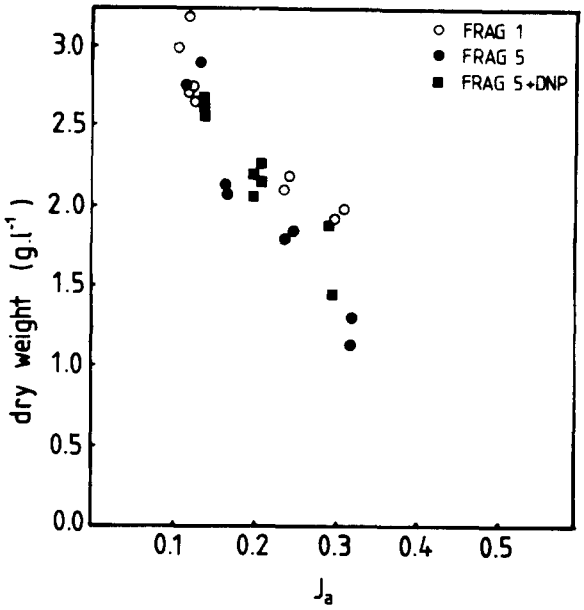

Fig. 2. Relationship between the bacterial dry weight and rate of growth (J_a = dilution rate). ○, FRAG 1; ●, FRAG 5; ■, FRAG 5 + DNP.

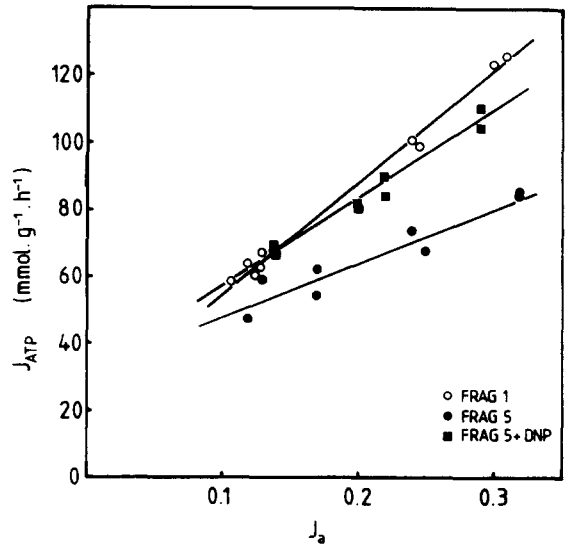

Fig. 3. Relationship between the ATP flux (J_{ATP}) and the rate of growth (J_a) of FRAG 1 (○), FRAG 5 (●) and FRAG 5 + 0.5 mM DNP (■).

quantitatively converted into CO_2, biomass and acetic acid. The specific consumption rate of glucose and the specific production rate of CO_2 increased with increasing growth rates. The specific production rate of acetic acid showed an increase with increasing growth rates in FRAG 1, and a decrease with increasing growth rates in FRAG 5. From these product-formation rates, the rate of ATP production can be calculated (see Materials and Methods). Fig. 3 shows that for each growth rate the rate of ATP production in FRAG 1 was higher than in FRAG 5. The two lines are described by (best fit by linear regression):

$$J_{ATP} = 332(\pm 11)\cdot(-J_a) + 22(\pm 2)\ \text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$$

for FRAG 1

and

$$J_{ATP} = 165(\pm 21)\cdot(-J_a) + 32(\pm 5)\ \text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$$

for FRAG 5.

With FRAG 5, also the effect of DNP on ATP production was determined: the results are plotted in the same figure and show that for all growth rates a higher ATP production rate was required to maintain the steady state.
The line describing these results is:

$$J_{ATP} = 262(\pm 16)\cdot(-J_a) + 31(\pm 3)\ \text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$$

Both FRAG 1 and FRAG 5 were also cultured under glucose-limited conditions. Because in this case potassium is no longer a limiting substrate, the high-affinity system (Kdp) will not be induced in FRAG 1 under these conditions. As expected, the bacterial dry weight was the same at all growth rates. Also the ATP production rates were identifical for both parent and mutant at each growth rate.

At different growth rates under K^+-limited conditions, we determined the distribution of potassium between medium and cells in the chemostat. It was found that the extracellular potassium concentration was very low (less than 25 μM) under all conditions, which would mean that virtually all potassium was present within the cells. The values were too low to allow precise calculation of the gradient. Taking into account the results of Fig. 2, this means that the intracellular potassium concentration rises with increasing growth rates [23,24]. Assuming an internal water

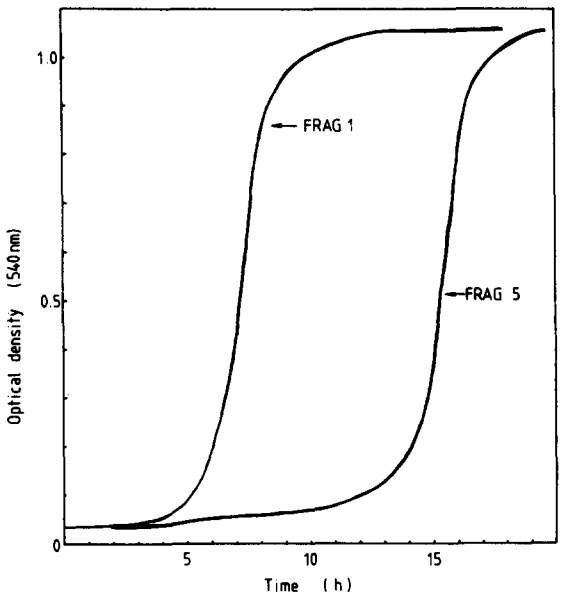

Fig. 4. Growth of FRAG 1 and FRAG 5 in batch culture, monitored as the increase in optical density at 540 nm.

space of 1.45 μl/mg dry weight [25], it can be calculated that the gradient of potassium across the membrane was 10 000 or more.

FRAG 1 and FRAG 5 were also grown in batch culture with limited amounts of potassium. Growth was monitored as the increase in optical density of the suspension (Fig. 4). Although the maximal growth rate (μ_{max}) of both strains did not differ very much, FRAG 5 had a much longer lag phase. The final optical density was the same for both strains, which means that also in batch cultures the yield on potassium was the same for the two strains (cf. Fig. 2).

Discussion

Under potassium-limited conditions, the ATP production rate that is required to maintain a steady state of growth, was higher for FRAG 1 than for FRAG 5. Apparently, FRAG 1 had more ATP dissipating processes than FRAG 5. We have postulated (see Introduction) that this might be due to an ATP-utilising futile cycle of potassium across the membrane due to the presence of more than one K^+-uptake system (Fig. 1). FRAG 5 lacks one of these systems (Kdp) so that the futile cycle is interrupted.

Comparison of the magnitude of the ATP-dissipating processes with the capacity of the potassium-uptake systems shows that at an intermediate growth rate of 0.2 h^{-1}, the difference in ATP flux between the two strains is 19 mmol $ATP \cdot g^{-1} \cdot h^{-1}$ (0.32 mmol $ATP \cdot g^{-1} \cdot min^{-1}$). The maximal rate of uptake of potassium via the Kdp system reported in the literature is 0.15 mmol $K^+ \cdot g^{-1} \cdot min^{-1}$ with a K_m of 0.002 mM [13]. For the Trk A system these values are 0.55 mmol $K^+ \cdot g^{-1} \cdot min^{-1}$ and 1.5 mM, respectively [13]. Although these figures were obtained with cells grown under different conditions from ours (e.g., the cells used by Rhoads et al. [13] were grown in batch culture and depleted before use of K^+, whereas our cells were grown in the chemostat under constant potassium-limited conditions), they may be indicative for our hypothesis. Under potassium-limited conditions, in FRAG 1 the high-affinity system will work at V_{max}, whereas the Trk system is only able to pump very slowly. According to our hypothesis, the Trk system will operate in reverse and will possibly do this at V_{max} (the internal K^+ concentration is very high). In FRAG 5, the capacity of the low-affinity system is of the right order of magnitude to be responsible for the growth behaviour observed in the chemostat.

To be able to calculate the stoichiometry between ATP utilisation and K^+ cycling, it would be necessary to measure this cycling under the exact conditions of the chemostat. Since the ATP dissipation through a futile cycle of potassium across the membrane is still not proved, we tested the influence of DNP on the ATP flux. This compound uncouples oxidative phosphorylation, which leads to ATP dissipation. Fig. 3 shows that the effect of DNP at the concentration used was, indeed, very similar to that of introduction of an extra potassium-uptake system.

Analysis of the data of Fig. 3 according to the MNET model (see Introduction) shows clearly that potassium limitation is equivalent to catabolite limitation. This can be concluded from the fact that the slope of the line relating J_{ATP} and $J_a (= D)$ increases upon introducing an 'ATP leak', either by a futile K^+ cycle or by DNP.

Apparently, growth at low potassium concentrations is not limited by incorporation of

potassium into the cell, but by the energetic demand imposed by the presence of the high gradient of potassium.

It would seem that the parent *E. coli* cells are at a disadvantage to grow under low potassium conditions compared to the strain lacking the high-affinity potassium uptake system (Kdp). However, as shown in Fig. 4, the parent can outdo the mutant during the initial phase after addition of a small amount of K^+. Presumably, under this condition the presence of more uptake systems outweighs the disadvantage of the futile cycle. It is only under conditions of steady state in the chemostat that the parent has to expend significantly more of the energy source than the mutant in order to survive.

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