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Control analysis of the dependence of *Escherichia coli* physiology on the H^+-ATPase

(*atp* operon/growth rate/yield/respiration/*lac* promoter)

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ABSTRACT **The H^+-ATPase plays a central role in *Escherichia coli* free-energy transduction and hence in *E. coli* physiology. We here investigate the extent to which this enzyme also controls the growth rate, growth yield, and respiratory rate of *E. coli*. We modulate the expression of the *atp* operon and determine the effect on said properties. When quantified in terms of control coefficients, we find that, in the wild-type cell growing on glucose in minimal medium, this key enzyme (H^+-ATPase) exerts virtually no control on growth rate ($|C| < 0.01$), a minor positive control on growth yield ($C = 0.15$), and a small but negative control on respiration rate ($C = -0.25$). The control the enzyme exerts on the consumption rate of the carbon and free-energy substrate is negative ($C = -0.15$). We also studied how the control coefficients themselves vary with the expression of the *atp* operon. As the level of expression of the *atp* operon was reduced, the control exerted by the H^+-ATPase on growth rate and growth yield increased slightly; the control on growth rate passed through a maximum ($C = 0.1$) and disappeared when the *atp* operon was not expressed at all, reflecting that with this substrate there are alternative routes for ATP synthesis. At elevated levels of the H^+-ATPase compared to the wild type, the control exerted by the enzyme on growth rate became negative. The evolutionary context of the absence of control by the *atp* operon on growth rate is discussed.**

When cells are cultured aerobically, much of the free-energy transduction from catabolism to endergonic cell processes is catalyzed by the H^+-ATPase of the energy-coupling membrane. In eukaryotes this membrane is the inner mitochondrial membrane; in prokaryotes, it is the plasma membrane. If the free-energy substrate for catabolism is fermentable, there is an alternative route for generation of ATP, through substrate-level phosphorylation. Indeed, deletion mutants of the *atp* operon in *Escherichia coli* can grow at fairly high growth rate with glucose as the growth substrate (1, 2). However, the low growth yield [in terms of g (dry weight)/mol of glucose] of such cells indicates that oxidative phosphorylation is a major route for generating ATP in the wild-type *E. coli* cell, also when glucose is the free-energy substrate.

The fact that the H^+-ATPase is a key enzyme in the mechanism of cellular free-energy metabolism, however, does not necessarily mean that under physiological conditions it is also in control of that metabolism or of the aspects of cell physiology that depend on the latter. When an enzyme is in strict control of the normal flux through a cellular process, this is usually taken to mean that the activity of that process (flux through that process) is proportional to the activity of that enzyme. Then, a 10% increase in the V_{max} of the enzyme leads to a 10% increase in the steady-state flux through the process. The flux control coefficient defined by metabolic control analysis, which is the more precise quantifier of control (3–6), then equals 1.

Crucial enzymes in terms of mechanism can have low-flux control coefficients (for review, see ref. 7). Relatively little control over oxidative phosphorylation in rat liver mitochondria resided in cytochrome oxidase, an indispensable enzyme for the process (8). It is therefore of interest to determine the extent to which the H^+-ATPase controls cell physiology. Eukaryotic cells are rather inaccessible to such studies. In *E. coli* however, a good method exists to measure control coefficients by enzymes. Basically, one brings the gene encoding the enzyme under the control of a promoter one can modulate from the outside, usually by varying the concentration of isopropyl β-D-thiogalactoside (IPTG) (9–11). The control coefficients equal the effects on physiological properties divided by the measured modulation of the enzyme concentration. Here we have modulated the expression of the chromosomal *atp* operon and measured the control that the H^+-ATPase exerts on *E. coli* physiology during aerobic growth with glucose as the growth substrate. Surprisingly, in view of the important role of the H^+-ATPase in cell physiology, we find that it exerts virtually no control on growth rate. Its control on respiration and substrate consumption is negative.

MATERIALS AND METHODS

Bacterial Strains. LM3118, the *E. coli* K-12 strain used as the wild-type strain in this study, has the genotype F^+, *asnB32, thi-1, relA1, spoT1, lacUV5*, and $lacY^{am}$. The wild-type promoters of the chromosomal *atp* operon have been replaced in the strain LM3112 with a single inducible *lacUV5* promoter and in the strain LM3113 with a single *tacI* promoter. Both strains carry the *lacY* and *lacUV5* mutations in the *lac* operon to allow for ready control and monitoring of the expression of the *atp* operon (12).

Growth of Bacterial Cultures. Cultures were grown at 30°C as described (12) in minimal Mops medium (13), supplemented with thiamine (1 μg/ml) and 0.011% glucose. Under these conditions the wild-type strain LM3118 had an actual growth rate of $\mu = 0.59\ h^{-1}$ (0.85 doublings per h) and the growth yield was 88.5 g (dry weight)/mol of glucose. To avoid mutational drift, LM3112 was stored [overnight culture diluted 1:1 with 50% (vol/vol) glycerol at −80°C] in the presence of 1 mM IPTG.

Respiratory Rates. Rates were determined as described (2). The respiration rate of the wild-type strain (LM3118) was 12.4 mmol of O_2 per h per g (dry weight).

Quantitation of c Subunit. This was done as described by von Meyenburg *et al.* (14) and Jensen *et al.* (12).

Curve Fitting and Control Coefficients. To calculate the control coefficients at the wild-type level of H^+-ATPase, we

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Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

used the IPTG concentration to modulate the *atp* operon expression in a limited range around the wild-type level (Table 1). Data points refer to growth rate, growth yield, respiratory rate, or substrate consumption rate at various concentrations of c subunit. Through corresponding data points a second-order polynomial was drawn, using a nonlinear least-squares fitting procedure (MLAB, Civilized Software, Bethesda). The control coefficient was then calculated as described below, at the level of c subunit where growth rate, respiratory rate, and growth yield corresponded to the wild-type values.

To estimate the control of the H^+-ATPase on the growth variables for the entire range of H^+-ATPase expression, we fitted smooth curves through the experimental data. The growth-rate fit was $\mu(c) = (27.7e^{-0.33c} - 34e^{-1.56c} + 84)$, the growth-yield fit was $Y(c) = [(55.3e^{-0.0089c})(1 - e^{-1.37c}) + 53.6]$, and the respiration rate was $J_o(c) = (52e^{-0.94c^2} + 85.9)$, where c is the concentration of H^+-ATPase c subunit in the cells. The glucose consumption data were fitted by $J_{glucose}(c) = [110(c + 0.1)^{-0.145} - 5]$. The functional forms used led to the closest nonlinear least-squares fits out of a number of functional forms tried. Other functional forms with small summed squared deviations gave results within the experimental error. The control coefficients for the entire expression range were calculated from the functions above, using the equation, $C_c^{F(c)} = c/F(c) \cdot dF(c)/dc \{= d\log[F(c)]/d\log(c)\}$, i.e., the percentage change in variable $[F(c)]$ divided by the percentage modulation of the H^+-ATPase concentration (c) in the limit of the latter percentage to zero (15).

RESULTS

The structural subunits of the *E. coli* H^+-ATPase are encoded by eight genes in the *atp* operon and transcribed from three promoters. To modulate the concentration of H^+-ATPase around its wild-type level, we used strains where the wild-type *atp* promoters on the chromosome have been replaced by single IPTG-inducible promoters (12). We grew these strains in the presence of various concentrations of IPTG and determined the respiratory rate of the cells and their growth rate during exponential growth at optical densities of <0.3 (λ = 450 nm). The expression of the *atp* genes was followed by measuring the relative concentration of the c subunit of the H^+-ATPase. The growth yield was determined from the final optical density for a complete culture initiated at a known limiting concentration of growth substrate, ending at optical densities of 0.2–0.3.

Control by the H^+-ATPase at its Wild-Type Level. When the *atp* operon was transcribed from the *lac*-type promoters, we observed (n = 3) that when the expression of c subunit was set at 1.4 times the wild-type level, the growth rate, the growth yield, and the rate of respiration were all very close to the wild-type values (Table 1). We take this to indicate that, at the same concentration of c subunit, the activity of the H^+-ATPases synthesized was smaller in the IPTG modulation strains than in the wild-type cells but that this phenomenon was not accompanied by enhanced uncoupling. Accordingly, we took 1.4 times c subunit to represent the expression level that is relevant for analysis of the control of H^+-ATPase at its wild-type level. We verified that had the control been calculated at the concentration of c subunit found in wild-type cells, the control coefficients would have been only marginally different.

To determine the coefficients of control for the H^+-ATPase, one has to change the concentration of this enzyme by a small fraction and measure the effect on the steady-state properties of interest. In theory the fractional change should be infinitely small, but this is not practicable. Instead, we choose to modulate the H^+-ATPase concentration over a small but finite range around its wild-type level. This then allowed us to determine a smooth curve for the dependence of the property under study on the H^+-ATPase concentration. The slope (or more precisely the log–log derivative) of this smooth curve at the wild-type level was then used to estimate the control coefficient.

For the range of 50% around that wild-type level (c subunit = 1.4), Table 1 shows how the growth rate, the growth yield, the respiration rate, and the rate of glucose consumption varied with the concentration of H^+-ATPase (as estimated from the concentration of the c subunit). We used a nonlinear least-squares method to fit a second-order polynomial to the experimental data. The control coefficients, given in Table 1, were then calculated at c subunit = 1.4. Clearly, at the wild-type level, the H^+-ATPase hardly controls growth rate on glucose ($C_c^{\mu} = 0.00$). It does control the growth yield somewhat ($C_c^{Y} = 0.15$). The control of the H^+-ATPase on respiration and glucose consumption is negative ($C_c^{J_o} = -0.25$ and $C_c^{J_g} = -0.16$).

The Control of Physiology by the H^+-ATPase at Reduced and Enhanced Expression Levels of the *atp* Operon. Most often, the control exerted by an enzyme increases when its activity is reduced greatly, and it decreases when the enzyme is overproduced. Our experimental tools allowed us to change the expression of the *atp* operon to a large extent and then modulate the expression again around that altered level. In practice this boils down to measuring the complete dependence of growth rate and growth yield on the concentration of H^+-ATPase for a wide range of concentrations of the latter. The *lacUV5* promoter is less active than the *tacI* promoter when fully induced, but more repressible (12, 16): in the absence of IPTG, the c subunit of the H^+-ATPase was not detectable [i.e., <5% of the wild-type level (12) when expressed from the *lacUV5* promoter] and the physiological variables measured were indistinguishable from the ones

Table 1. Control by the H^+-ATPase on *E. coli* physiology

Exp.		Relative rate, yield (%)								Control coefficient
1	c subunit	0.62	0.77	1.03	1.38	1.63	1.97			—
	Growth rate (μ)	95.0	95.8	97.3	99.9	98.7	96.1			0.00 (0.01)
	Growth yield (Y)	81.3	87.6	93.9	99.4	103.6	104.8			0.15 (0.02)
	$J_{glucose}$	117	109	103	100	95	92			−0.16 (0.03)
2	c subunit	0.58	0.91	1.04	1.31	1.47	1.70	1.84	2.16	—
	Respiration (J_o)	122	107	106	101	87	80	91	89	−0.25 (−0.23 to −0.29)

Expression of H^+-ATPase c subunit is expressed as units relative to the amount found in wild-type cells. The data from experiment 1 are from six individual cultures with increasing IPTG concentrations (15, 18, 21, 25, 30, and 35 μM), and data from experiment 2 are from eight cultures with increasing IPTG concentrations (15, 18, 20, 22.5, 25, 27.5, 30, and 40 μM). This representation was chosen because, although a certain level of c subunit can be maintained throughout an experiment, it is difficult to reproduce exactly the same level of c subunit (which would also be irrelevant in the present context). The control coefficients were calculated at 1.4 times the wild-type expression of c subunit (see also text) by using the experimental data for the relative rates. The accuracy was estimated from the results of three or four independent experiments; the values in parentheses represent the maximum deviation from the control coefficient listed. The accuracy of the control coefficient for the respiration rate was estimated by removing the data points one by one and recalculating the coefficient.

obtained with a strain carrying a complete deletion of the *atp* genes. Using the two promoters, we were thus able to vary the expression of the H^+-ATPase c subunit from essentially zero up to 4.5 times the wild-type level.

Fig. 1 *A* and *B* shows how the growth rate and growth yield varied with the concentration of the H^+-ATPase. As the concentration of H^+-ATPase was increased from virtually zero to the wild-type level (1.4 times for the c subunit), the growth rate increased gradually from 80% to 100%. In the same expression range, the growth yield increased from 50% to 100%. As the expression of the *atp* operon was increased above the wild-type level, the growth rate decreased slightly, and at 4.5 times the wild-type expression level, the growth rate was 89% of the wild-type rate. In the same expression range, the growth yield increased to 106%.

The control exerted by the H^+-ATPase on growth rate (Fig. 1*C*) and growth yield (Fig. 1*D*) increased as the expression of the *atp* operon was decreased, until a maximum was reached, after which the control decreased to zero. This secondary decrease reflects the phenomenon that because of substrate-level phosphorylation, growth does not completely depend on the H^+-ATPase.

When the *atp* operon was overexpressed with respect to the wild-type level, the control on growth rate became negative, whereas that on growth yield approached zero.

Control on Respiration and Substrate Consumption Rate. We also analyzed how the respiration rate depended on the H^+-ATPase for the complete range between 0 and 4.5 times the wild-type level (Fig. 2*A*). As the expression increased from zero to the wild-type level, the respiration rate decreased from 140% to the wild-type level. When the *atp* operon was overexpressed, the respiration rate dropped to 90% of the wild-type rate. In terms of control coefficients, the control exerted by the H^+-ATPase on respiration was negative around the wild-type expression level and both at very low and at very high enzyme concentrations, the control approached zero (Fig. 2*C*). Of course, the basis for this phenomenon is that, as the concentration of the H^+-ATPase tended toward zero, respiratory rate did not.

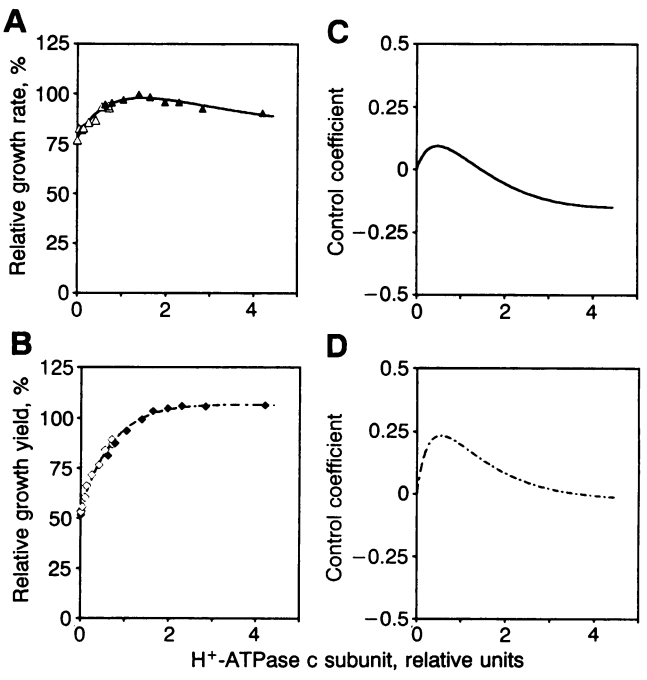

FIG. 1. Control by the H^+-ATPase on *E. coli* growth. Variation of growth rate (*A*) and growth yield (*B*) as a function of the expression of H^+-ATPase c subunit. *E. coli* cells were used, in which the expression of the *atp* operon could be controlled by either the *lacUV5* promoter (strain LM3112; open symbols) or the *tacI* promoter (strain LM3113; solid symbols), through various concentrations of IPTG. Smooth curves were drawn through the experimental data points. (*C* and *D*) Control of H^+-ATPase on *E. coli* growth rate and yield, respectively. The log–log derivatives of the smooth curves from *A* and *B* were plotted as a function of the level of H^+-ATPase.

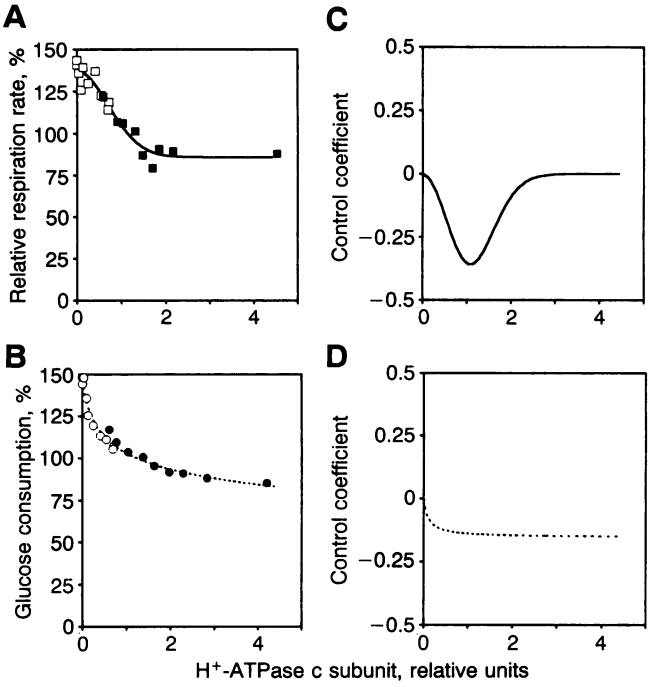

FIG. 2. Control by the H^+-ATPase on *E. coli* catabolism. The rate of respiration (*A*) and glucose consumption (*B*) was plotted as a function of the level of H^+-ATPase. The rate of glucose consumption was calculated from experimental measurements of growth rate and growth yield. Open symbols, strain LM3112 (*atp*::*lacUV5p*); solid symbols, strain LM3113 (*atp*::*tacIp*). (*C* and *D*) Control of H^+-ATPase on the rates of respiration and glucose consumption, respectively. The log–log derivatives of the smooth curves on *A* and *B* were plotted as a function of the level of H^+-ATPase.

The observation that, with decreasing concentrations of the H^+-ATPase, the growth yield decreased while the growth rate remained virtually constant implied that the rate of glucose consumption changed as the H^+-ATPase concentration was modulated ($J_{glucose} = \mu/Y$). As shown in Fig. 2*B*, the rate of glucose consumption increased at decreasing concentration of H^+-ATPase and was increased to 140% at zero concentration of H^+-ATPase. When the H^+-ATPase was overexpressed, the rate of glucose consumption gradually decreased to 80% of the wild-type rate (at 4.5-fold overexpression of H^+-ATPase c subunit). Also for the control exerted by the H^+-ATPase on the rate of glucose uptake, we calculated the control coefficient, as shown in Fig. 2*D*. The value was small and negative throughout the entire expression range.

DISCUSSION

In this paper we have examined the extent to which the H^+-ATPase controls various growth variables in *E. coli*, growing on a fermentable substrate (glucose). We have done this by modulating the expression of the *atp* operon around the wild-type activity.

The dependence of growth rate on H^+-ATPase concentration exhibited a maximum close to the wild-type level of the enzyme (Fig. 1*A*). This result is in line with the results obtained by von Meyenburg *et al.* (1, 14). These investigators measured the growth rate of strains that either had a deletion in the *atp* operon or overproduced the H^+-ATPase from plasmids. Both the strain that had a deletion in the *atp* operon

and the strains that overexpressed the H^+-ATPase had decreased growth rates, indicating there is an optimal concentration of this enzyme in between. From those experiments however, it was not possible to conclude whether the H^+-ATPase concentration was optimal with respect to growth rate at the wild-type level or somewhat above or below that level. We observed that the control by the H^+-ATPase on growth rate was close to zero essentially at the wild-type level. A control coefficient of zero for growth could imply that the concentration of the enzyme in wild-type *E. coli* is optimal for growth rate. The differences between the results of von Meyenburg *et al.* (1, 14) and us, though subtle, are relevant when discussing the question for which functions *E. coli* has been optimized.

Growth yield is related to the coupling between growth and catabolism. Consequently, whether growth yield is optimal in wild-type *E. coli* is a distinct question; the cells may well sacrifice growth yield to grow as fast as possible (15). Our results suggest that this may be the case; only concentrations of H^+-ATPase higher than wild type are optimal with respect to growth yield. Around the wild-type level growth yield still increased with the concentration of H^+-ATPase. This reflects the phenomenon that at the wild type level the enzyme still exerts some control on the rate of substrate consumption.

Not only does the metabolic control theory suggest the flux control coefficient as the quantifier of the extent to which an enzyme in a metabolic or free-energy transduction pathway controls the pathway flux, it also offers explanations for the magnitude of these flux control coefficients (5, 15). In essence, it relates the flux control coefficient of any enzyme on any flux to the so-called elasticity coefficients of that enzyme for metabolic intermediates, taken relative to the elasticity coefficients of the other enzymes in the pathway toward the same metabolic intermediates. The elasticity coefficient, which is defined as the percentage increase in enzyme rate resulting from a 1% increase in concentration of the metabolic intermediate, is comparable to the Hill coefficient. For simple enzymes the elasticity coefficient is close to 1 when the metabolic intermediate is below the Michaelis constant and close to 0 when its concentration is far above the Michaelis constant. In isolated rat-liver mitochondria, it was indeed demonstrated that the combined control exerted by the adenine nucleotide translocator plus the H^+-ATPase on mitochondrial respiration could be explained from their elasticity toward the proton gradient taken relative to the elasticity of the respiratory chain for the proton gradient (15, 17). When an ATP-consuming system was present, the control by the adenine nucleotide translocator depended on the elasticity of the ATP-consuming system with respect to extramitochondrial ATP (18). One explanation therefore of our findings could be that the elasticity coefficient of the H^+-ATPase for the transmembrane electrochemical proton gradient is high in *E. coli* when compared to the elasticity of the respiratory chain for that gradient. That the elasticity of the respiratory chain with respect to the proton gradient may be very low in growing *E. coli* cells is consistent with (though not quite proven by) the observation that uncoupler-induced respiratory control is virtually absent (19, 20).

Hierarchical control theory (21, 22) has shown that in systems where regulation may involve regulated gene expression in addition to metabolic regulation, the elasticity coefficient should effectively also include interaction through the gene expression. Thus, the low control found for the H^+-ATPase could be due to a strong dependence of the expression of the genes encoding the respiratory chain on the intracellular level of ATP or on membrane potential. Indeed, we have found that an *atp* deletion mutant had an increased level of cytochromes (2).

It may be useful to compare the control exerted by the H^+-ATPase in *E. coli* to the control of the enzyme on active oxidative phosphorylation in rat liver mitochondria. In the latter case the control by the enzyme on respiration is also rather small but positive (23), and the translocator for adenine nucleotides usually exerts a significant positive (30%) control (8). In the *E. coli* cells, the control exerted by the H^+-ATPase on respiration is negative. This is a surprising difference, as a decrease in the amount of H^+-ATPase in the cell should be expected to lead to an increase in membrane potential (2). At increased membrane potential, the respiration rate is expected to decrease, and the control on respiration exerted by the H^+-ATPase is, therefore, expected to be positive. An important distinction, however, between isolated mitochondria and intact cells is that in the former case the redox pressure at which electrons are fed into the respiratory chain is kept constant experimentally, whereas in the latter case that redox pressure may vary. Since, in the intact cells, ADP has a stimulatory effect on glycolysis, one might expect that upon reduction of the H^+-ATPase concentration, the ensuing increase in ADP (2) would activate glycolysis and lead to an increased redox state of the cells, which in turn would activate respiration and thus account for the negative control exerted on respiration. The negative control on the rate of substrate consumption may be explained in the same way. The effect we noted on the expression of cytochromes (2) would provide an additional explanation.

In yeast the plasma membrane H^+-ATPase exerts a strong control on growth rate (11). The metabolic role of this H^+-ATPase, however, is quite different, not lying on the main route of free-energy transduction but serving to control the intracellular pH.

The sum of the control coefficients with respect to a flux must equal 1 (5). With respect to yield, the sum of the control coefficients should equal 0 (15, 24). Therefore, the finding of a low control on yield may be less surprising than the finding of a low control on growth rate. What may be more surprising is the finding that the control on yield exceeds the control on growth rate. Since the control on yield is mathematically equal to the control on growth rate minus the control on substrate consumption rate (24), the reason is that the H^+-ATPase exerts a negative control on the rate of substrate consumption, which has been discussed above.

In other systems it has been shown that the magnitude of control coefficients depends on the conditions imposed on the system (8, 15). We found this reflected in the observations (*i*) that the control coefficients differed somewhat between glucose and succinate (25) as growth substrate and (*ii*) that they varied when the expression level of the *atp* operon was changed. Roughly the changes in the control coefficients were in the expected direction: on succinate (where the H^+-ATPase is essential for growth) the *atp* operon had more of the control it already had when glucose was the substrate and the control by the H^+-ATPase increased (in absolute terms) as the expression level of the *atp* operon was decreased.

Some observations are perhaps less in line with expectations. The first is that, as the expression of the *atp* operon is decreased, the control on growth rate starts to increase toward 1 (as expected) but then returns to 0. The explanation for this is that there are two routes that synthesize ATP and we are considering only the control of one route on the total flux. Once much of the oxidative phosphorylation capacity has been eliminated by reducing the expression of the *atp* operon, the other route, substrate-level phosphorylation, becomes dominant and the route of oxidative phosphorylation has little control on the total flux. From this argument one would expect that when succinate is the growth substrate (i.e., when there is much less substrate-level phosphorylation), the control on growth should approach 1 as the level of the H^+-ATPase is reduced. This was indeed observed (25).

When the expression of the *atp* operon was increased above the wild-type level, the control on growth rate became negative. We have shown (25) that overexpression of H^+-ATPase results in an increased [ATP]/[ADP] ratio, and the negative control on growth rate might, therefore, be explained by a lower concentration of ADP (at high [ATP]/[ADP] ratios), e.g., by inhibition of phosphofructokinase and, hence, the production of building blocks for more biomass.

A major observation in this paper is the lack of control by the H^+-ATPase on growth rate at the wild-type level, and above we have discussed the possible mechanistic basis for this phenomenon. A high elasticity of the enzyme with respect to $\Delta\mu_{H^+}$ and/or the ATP/ADP ratio will cause its control to be low (15). Because, when defined in terms of flux control coefficients, total control on growth rate must be equal to 1, this implies that the control must reside in other processes, such as respiration or anabolism. A biologically interesting aspect, however, is why *E. coli* should have developed one of those mechanistic bases so as not to be controlled in its growth rate by the H^+-ATPase. The relationship between evolutionary pressure and the magnitude of control coefficients has been discussed by various authors [for review, see Heinrich *et al.* (26)]; enzymes with high control coefficients on growth rate are expected to evolve more quickly until their control coefficients are small. If this line of argumentation is followed, all enzymes would evolve up to a control coefficient that is inversely proportional to their possibility to increase in activity by further evolution. However, these considerations presumed that there would be no evolutionary advantage in having a particular distribution of control, which may be an oversimplification. In line with analyses presented elsewhere (15), we expect evolution to have focused the control into the processes that are most closely connected with physiological signals. For *E. coli* this suggests that control should perhaps lie in substrate uptake, hence not in the H^+-ATPase; the absence of control by H^+-ATPase we observed would fit such an interpretation.

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