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ATP-DEPENDENT PROTON TRANSLOCATION AND QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE IN INSIDE-OUT MEMBRANE VESICLES OF A CYTOCHROME-DEFICIENT MUTANT OF *ESCHERICHIA COLI*

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Summary

1. ATP-dependent proton translocation and ATP-dependent quenching of the fluorescence of 9-aminoacridine were measured in inside-out vesicles derived from a cytochrome-deficient mutant of *Escherichia coli*.

2. ATP-dependent quenching of fluorescence was inhibited by nigericin gramicidin, NH_4Cl, and carbonylcyanide-*m*-chlorophenylhydrazone. Inhibition was also produced by the ATPase inhibitors *N,N'*-dicyclohexylcarbodiimide (DCCD) and diphenyl phosphorazidate (DPA), and by the respiratory chain inhibitors piericidin A, 2-heptyl-4-hydroxyquinoline *N*-oxide, and Zn^{2+}. The inhibition of ATP-dependent fluorescence quenching by the ionophores, uncouplers, and respiratory chain inhibitors was not due to an effect on ATPase activity which was insensitive to these agents.

3. By use of the ATPase inhibitors DCCD and DPA, or by replacing ATP with GTP, ITP and CTP, a correlation between the ATPase activity and the rate of ATP-dependent membrane energization, as measured by fluorescence quenching, was obtained.

Introduction

The membranes of chloroplasts, mitochondria and bacterial cells can be energized by ATP hydrolysis through the membrane ATPase complex by the formation of a transmembrane proton gradient [1,2]. The direction of proton translocation is outwards in intact mitochondria and bacteria, as well as in right-side-out vesicles prepared from these sources. In contrast, the direction of

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DPA, diphenylposphorazidate; HOQNO,2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonate.

proton translocation is inwards with inside-out vesicles.

In studies of ATP-dependent membrane energization in *E. coli* the relationship between the activity of the ATPase and the rate of membrane energization has not been investigated. However, we have previously shown that there is a direct correlation between the rate of membrane energization and the rate of electron flow through the respiratory chain [3]. In the latter experiments membrane energization was measured by the extent of quenching of the fluorescence of 9-aminoacridine. Although there is still some disagreement about the exact mechanism of quenching of the fluorescence of this dye, much evidence supports the view that quenching occurs when the dye enters the vesicles in response to the formation of a transmembrane pH difference [4,5]. As discussed later, this technique is a more convenient and sensitive method for measuring ATP-dependent proton translocation than that which involves direct measurement of pH changes in the external medium [6,7].

In the present paper we have shown that the rate of membrane energization is dependent on the activity of the ATPase as measured by the rate of nucleoside triphosphate hydrolysis. The proton-translocating nature of this system was further confirmed by the use of inhibitors. The inside-out membrane vesicles used in this study were prepared from a cytochrome-deficient mutant of *E. coli* in order to avoid possible complications arising from the presence of a functional respiratory chain.

Materials and Methods

Chemicals

All chemicals were of reagent-grade purity. Disodium ATP and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) were obtained from Sigma Chemical Company. Gramicidin D, valinomycin, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (Calbiochem), 9-aminoacridine hydrochloride (Aldrich Chemical Company), *N,N'*-dicylclohexylcarbodiimide (DCCD) (Eastman Organic Chemicals) were purchased as indicated. Nigericin, piericidin A, and diphenyl phosphorazidate were generous gifts from Drs. R.J. Hosley (Lilly Research Laboratories), S. Tamura (University of Tokyo), and Y. Anraku (University of Tokyo), respectively.

Organism and growth

E. coli SASX76 (*F*$^-$, *hem A*$^-$, *met*$^-$, *trp*$^-$, *lac*$^-$, *str*$^-$) [8], a generous gift of Dr. A. Sasarman (University of Montreal, Canada), was grown aerobically at 37° C in 2 l flasks containing minimal salts-glucose medium [9] supplemented with 0.5% bacto-tryptone (Difco). The cells were harvested at the end of the exponential phase of growth. The cells were suspended in 50 mM Tris/H_2SO_4 buffer, pH 7.8, containing 10 mM $MgCl_2$ (1 g wet weight per 10 ml buffer) and disrupted in a French press (Aminco) at 20 000 lb/inch^2. The suspension was centrifuged at 17 000 × *g* for 10 min to remove whole cells and large cell fragments. The inside-out membrane vesicles were obtained by centrifuging the supernatant from the above step at 120 000 × *g* for 2 h. The resultant pellet was suspended at a concentration of 10—15 mg protein per ml in 300 mM KCl, 5 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonate

(HEPES)/KOH buffer, pH 7.5, or 150 mM KCl, 5 mM $MgCl_2$, 3 mM glycylglycine buffer, pH 6.2, for the fluorescence quenching and proton uptake experiments, respectively.

Quenching of the fluorescence of 9-aminoacridine

Fluorescence quenching was measured in a buffer containing 300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES/KOH buffer, pH 7.5, as previously described [3]. Fluorescence was measured at 22°C with a Turner model 420 spectrofluorometer connected to a Varian strip chart recorder. Fluorescence was excited by light at 420 nm and emission was measured at 500 nm. The concentrations of ATP, 9-aminoacridine and membrane particles used in the assays are given in the legends to the figures.

The transmembrane pH difference (ΔpH) was calculated from the fluorescence data by the method of Schuldiner et al. [10] using the equation as given by Bamberger et al. [11]:

$$\Delta pH = \log \frac{Q}{V(1 - Q)}$$

where Q is the fraction of the total fluorescence that was quenched, and V is the fraction of the volume of the osmotic compartment of the total volume. The value of 0.5 μl/mg protein was taken for the intravesicular volume [3].

Protein was determined by the Folin method [12].

ATP-dependent proton translocation

ATP-dependent proton translocation in inside-out membrane vesicles was measured by the method of West and Mitchell [6] except that all operations were carried out under aerobic conditions. The output from a Beckman Model 310 expanded scale pH meter was measured on a Sargent-Welch model SRG recorder operating so that a full scale (24 cm) deflection was equivalent to 0.2 pH units.

ATPase activity

ATPase activity was measured either by estimating the release of inorganic phosphate from ATP [13] or from the change in pH accompanying the hydrolysis of ATP [14].

Results and Discussion

ATP-dependent proton translocation in cytochrome-deficient inside-out vesicles

We have previously shown that uncoupler-sensitive active transport of sugars and amino acids and energy-dependent transhydrogenation of $NADP^+$ by NADH can be driven by ATP hydrolysis in cytochrome-deficient whole cells and inside-out vesicles, respectively [9,15]. Uncoupler-sensitive, ATP-dependent proton translocation in inside-out vesicles prepared from cytochrome-deficient cells is shown in Fig. 1. Measurements of this type are complicated by the fact that ATP hydrolysis itself results in the production of protons. In order to correct for this process the reaction (Fig. 1, curve 2) was also carried

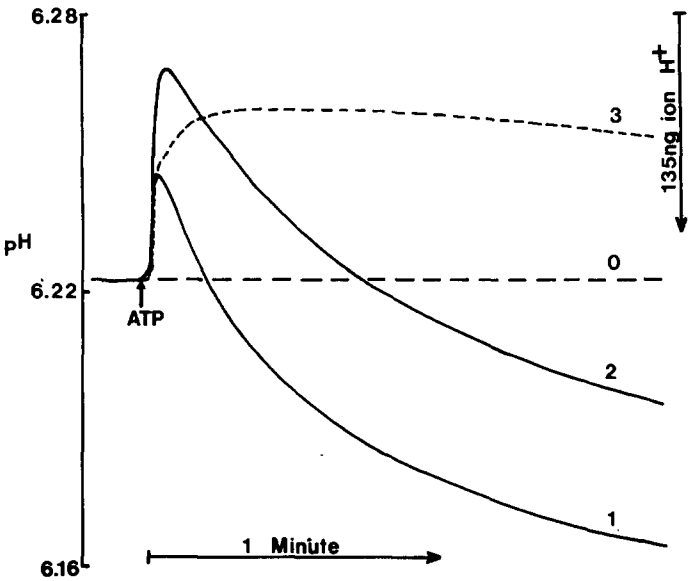

Fig. 1. Strip-chart recordings of changes in external pH following addtion of ATP to suspensions of cytochrome-deficient inside-out membrane vesicles. The reaction mixture contained in a total volume of 3 ml, 2.4 mg membrane protein, 3 μg valinomycin, 5 mM $MgCl_2$, 150 mM KCl, and 3 mM glycylglycine buffer, pH 6.2. The reaction was initiated by the addition of 0.5 mM ATP. In experiment 1 25 μM CCCP was also present. Curve 3, which measures the net change in the pH of the medium due to the uptake of protons into the vesicles, was calculated as the difference, curve 2 minus curve 1, plotted relative to the baseline, curve 0.

out in the presence of the uncoupler CCCP which abolished the uptake of protons into the vesicles (Fig. 1, curve 1). The difference between the two curves, with and without uncoupler, measured the extent of the uncoupler-sensitive proton uptake by the vesicles (Fig. 1, curve 3). No proton translocation was observed when the experiment was carried out in the presence of the ATPase inhibitor, DCCD. The reason for the initial rapid phase of alkalinization following addition of ATP, which occurred both in the presence and absence of CCCP, is not clear since the pH of the medium and of the ATP were adjusted to the same value. A similar response can be seen in an experiment of this type by Thayer and Hinkle [16] in which beef-heart submitochondrial particles were used.

Effect of inhibitors on ATP-dependent quenching of the fluorescence of 9-aminoacridine

Besides its sensitivity and convenience, the fluorescence technique was preferred to that involving a direct measurement of the pH change since the reaction could be carried out at pH 7.5 instead of pH 6.1—6.2. The latter pH range, although optimal for the direct measurements, is well below the optimal pH of 7.5—8.5 for ATP hydrolysis by the inside-out membrane vesicles [17]. Moreover, the fluorescence measurements did not require correction for the effect of proton production by the hydrolysis of ATP itself. This advantage was important for the determination of the kinetics of ATP-dependent energization.

The addition of ATP resulted in the quenching of up to 60% of the fluorescence of 9-aminoacridine in the presence of inside-out membrane vesicles from cytochrome-deficient cells of *E. coli* SASX76. The addition of nigericin inhibited fluorescence quenching by ATP (Figs. 2 and 3). Since this occurred in KCl, but not in NaCl-containing buffer, the inhibition of quenching was probably due to the decrease in the pH difference across the membrane produced by the electro-neutral exchange of K^+ for H^+. The addition of valinomycin was able to partly reverse the inhibition produced by nigericin. The ability of valinomycin to reverse the effect of nigericin was influenced by the relative concentrations of the inophores. At weight ratios of valinomycin to nigericin of less than 20 : 1 valinomycin could not reverse the inhibition of quenching by nigericin. Presumably, at the higher ratios the rate of K^+ extrusion mediated by valinomycin in response to the membrane potential would be greater than the rate of entry of K^+ through the electroneutral exchange with H^+ mediated by nigericin. Thus, the pH difference could reform. Conversely, at the higher levels of nigericin the rate of extrusion of K^+ in the presence of valinomycin would be lower than its rate of entry mediated by nigericin. Consequently, the pH difference could not reform. These results, and their interpretation, are similar to those previously obtained for the quenching of 9-aminoacridine fluorescence by substrate oxidation through the respiratory chain [3,4]. The inhibitory action of gramicidin, and of the uncouplers CCCP and ammonium ions, confirmed that ATP-dependent fluorescence quenching was due to the formation of a transmembrane pH difference (Fig. 3). None of these compounds inhibited ATPase activity, and at a concentration of 2 μg gramicidin/ml a 30—40% stimulation of ATPase activity was observed.

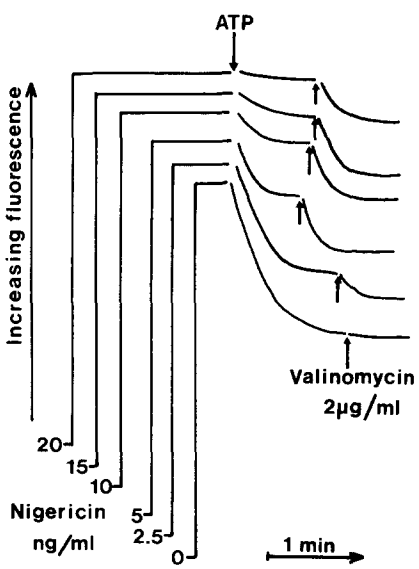

Fig. 2. Inhibition by nigericin of ATP-dependent quenching of the fluorescence of 9-aminoacridine in cytochrome-deficient inside-out membrane vesicles, and its reversal by valinomycin. Membrane vesicles (0.8 mg protein/ml) were preincubated with various concentrations of nigericin at 22°C for 2 min before fluorescence quenching was initiated by the addtion of 0.25 mM ATP (dipotassium salt). Valinomycin (2 μg/ml) was added where indicated.

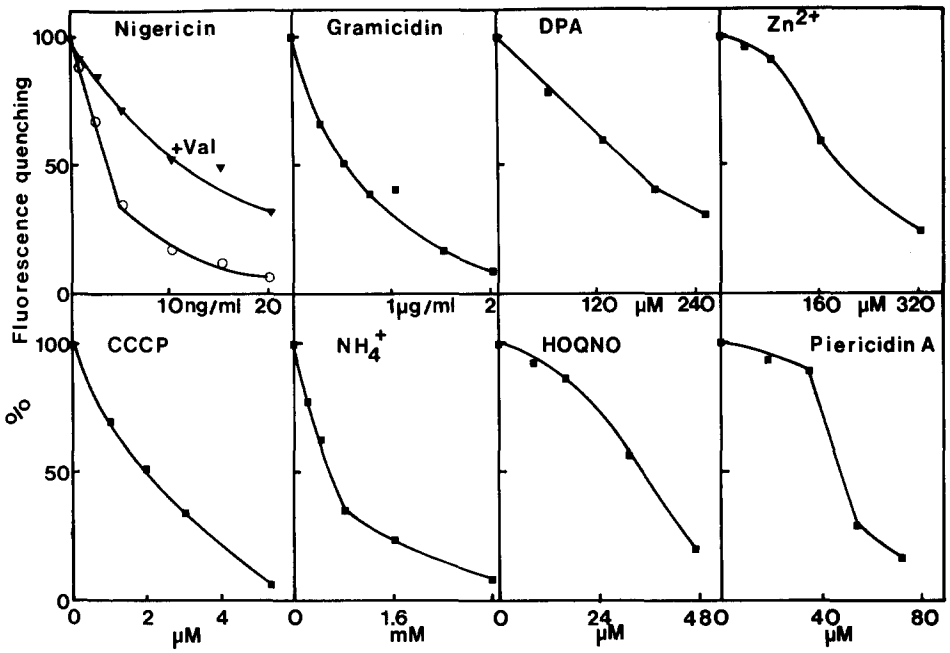

Fig. 3. Effect of ionophores, uncouplers, and ATPase and respiratory chain inhibitors on the ATP-dependent quenching of the fluorescence of 9-aminoacridine in cytochrome-deficient, inside-out membrane vesicles. The reaction mixture contained 0.8 mg/ml membrane protein, 5 µM 9-aminoacridine, 300 mM KCl, and 5 mM $MgCl_2$ in 10 mM HEPES/KOH buffer, pH 7.5, except for experiments with DPA and CCCP where 1.6 mg/ml membrane protein and 10 µM 9-aminoacridine were present. The reaction was initiated by the addition of 0.5 mM ATP. + Val, 1 µg/ml valinomycin present. The membrane vesicles were preincubated for 4 min at 22°C in the presence of the inhibitors.

We have calculated the transmembrane pH difference from the extent of fluorescence quenching by the method of Schuldiner et al. [11]. In the absence of nigericin a pH difference of 3.3 units was found which was reduced to 2.5 units in the presence of 20 ng nigericin/ml. Although this value is higher than the maximum transmembrane pH difference of 2 units previously found in *E. coli* [18], a minimum pH difference of this size was required to demonstrate net ATP synthesis in cells in the presence of an artificially-imposed pH gradient [19].

The ATP-dependent quenching of 9-aminoacridine fluorescence was inhibited by the ATPase inhibitors [17,20] DCCD and DPA (Figs. 3 and 4). This agrees with work using mutants having defective ATPase activity which has shown that ATP is utilized through the membrane ATPase for ATP-dependent quenching of fluorescence [21]. However, the inhibitory effect of the respiratory chain inhibitors Zn^{2+}, HOQNO and piericidin A [22,23] on ATP-dependent quenching in cytochrome-deficient vesicles cannot be explained by an effect on the ATPase, which is insensitive to these compounds, or by inhibition of oxidase activity since this is absent in the cytochrome-deficient cells (Fig. 3). Under these conditions the compounds seem to act like uncouplers although the lack of structural similarities to other uncouplers makes this doubtful. Since these compounds have a site of action in the respiratory chain

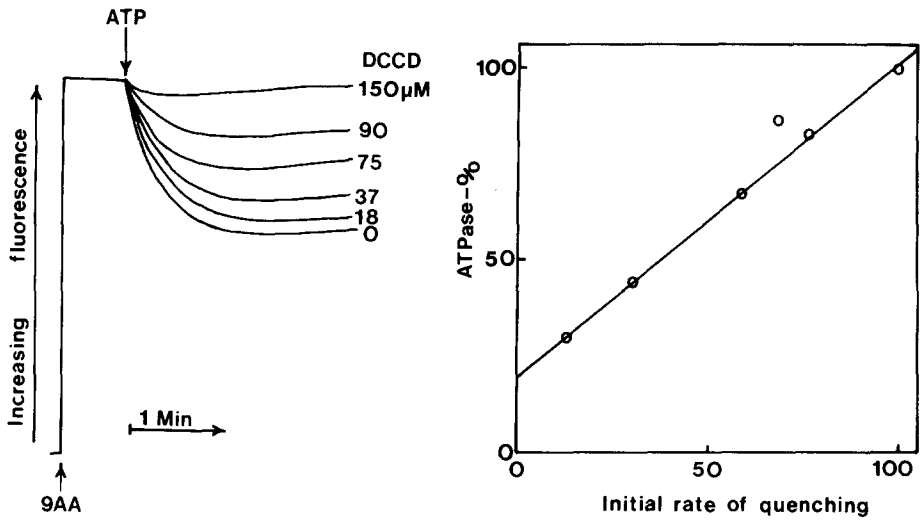

Fig. 4. Correlation between ATPase activity in the presence of DCCD and the initial rate of ATP-dependent quenching of the fluorescence of 9-aminoacridine in cytochrome-deficient, inside-out membrane vesicles. The initial rate of fluorescence quenching in the presence of different levels of DCCD obtained from the experiment shown on the left-hand side of the figure is plotted versus the ATPase activity of the vesicles measured under the same conditons. Fluorescence measurements were carried out as described in the legend to Fig. 3 with 1 mg membrane protein/ml. ATPase activity was measured by following the release of H^+. Both ATPase activity and the initial rate of fluorescence quenching are expressed as percentages of the values obtained in the absence of DCCD.

of the wild-type strain [22,23], it is possible that by binding to the non-functional respiratory chain of the cytochrome-deficient mutant, these inhibitors might alter either the membrane structure itself or might effect an association of the ATPase complex with the respiratory chain in such a way that fluorescence quenching would be inhibited without there being any effect on the ATPase activity. The sigmoidal nature of the inhibition curves obtained with these inhibitors (Fig. 3) could be explained, as for the action of antimycin A on the mitochondrial respiratory chain [24], as indicating the cooperative nature of the inhibition and implying that the inhibitor induced conformational changes in the system involved in fluorescence quenching. This would be consistent with the above hypotheses. The uncoupler-like action of these compounds has been observed in other *E. coli* systems. Hirata et al. [25] found that 50 μM HOQNO inhibited the uptake of proline into right-side-out membrane vesicles driven by an artificially-imposed membrane potential. There was little effect at an inhibitor concentration of 20 μM. Butlin et al. [26] observed that 130 μM piericidin A caused complete inhibition of ATP formation during oxidative phosphorylation by inside-out vesicles of *E. coli* but inhibited oxidation by only 43%. Zn^{2+} uncoupled the energy dependent transhydrogenase reaction in membrane vesicles of *Salmonella typhimurium* [27].

Correlation between ATPase activity and the rate of fluorescence quenching

There have been no previous reports on a correlation between the ATPase activity and the rate of ATP-dependent membrane energization although this might be predicted. Two methods were employed to show this relationship.

The rate of membrane energization was measured by the rate of fluorescence quenching in the presence of ATP. ATPase activity was regulated either by the use of the ATPase inhibitors DCCD and DPA or by using other nucleoside triphosphates besides ATP.

The effect of different concentrations of DCCD on the ATP-induced quenching of the fluorescence of 9-aminoacridine is shown in Fig. 4. Incubation of inside-out membrane vesicles with various concentrations of DCCD resulted in varying degrees of inhibition of ATPase activity and ATP-dependent fluorescence quenching. Both the initial rate and the steady-state level of quenching were affected by DCCD. Similar results were obtained with DPA. A plot of the initial rate of fluorescence quenching versus ATPase activity in the presence of DCCD was linear (Fig. 4). The extrapolated line did not pass through the origin. This may be due to the presence of DCCD-insensitive ATPase activity which is not linked to membrane energization because it has been dislocated from the DCCD-sensitive site in the membrane, or to the presence of another ATP-hydrolyzing enzyme.

As we have previously observed, the rate of hydrolysis of triphosphates by the ATPase is in the order ATP > GTP > ITP > CTP [13]. As shown in Fig. 5 the rate of quenching of the fluorescence of 9-aminoacridine by these nucleoside triphosphates was directly proportional to their rate of hydrolysis by the ATPase. Ferguson et al. [28] using the anionic fluorescence probe 1-anilinonaphthalene-8-sulfonate showed that its rate of fluorescence enhancement on energization of submitochondrial particles was lower with ITP than with ATP in qualitative agreement with the relative rates of hydrolysis of the two nucleoside triphosphates. In contrast to our results, there was no relationship between

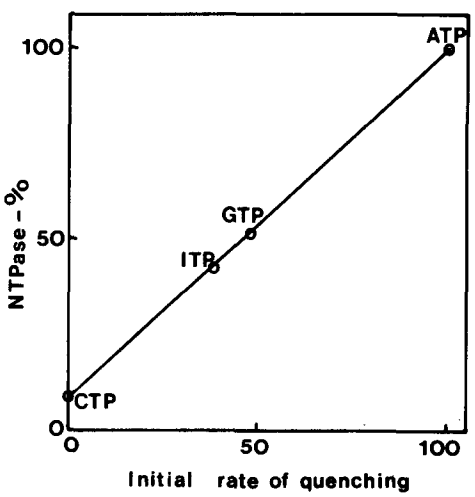

Fig. 5. Correlation between nucleoside triphosphatase (NTPase) activity and the initial rate of nucleoside triphosphate-dependent quenching of the fluorescence of 9-aminoacridine in cytochrome-deficient, inside-out membrane vesicles. Nucleoside triphosphate-dependent quenching of fluorescence was measured as described in the legend to Fig. 3 with 1 mg membrane protein/ml. Nucleoside triphosphatase activity was measured under the same experimental conditions by following the rate of release of inorganic phosphate. Both nucleoside triphosphatase activity and the initial rate of nucleoside-dependent fluorescence quenching are expressed as percentages of the values given by ATP.

the rate of fluorescence change and the activity of the ATPase in submitochondrial particles when this was varied with DCCD. However, this probe and 9-aminoacridine do not respond to the same parameters of the energized state. Thus, 9-aminoacridine responds primarily to the transmembrane pH difference [4,5] whereas 1-anilino-naphthalene-8-sulfonate appears to respond to both the pH difference and the membrane potential [29].

Our results indicate that the rate of energization of the membrane, and so, presumably, the rate of proton translocation, is directly dependent on the rate at which ATP and other nucleoside triphosphates are hydrolyzed by the ATPase. Thus, the regulation of ATPase activity would present an ideal means of controlling membrane energization. At present, there is no convincing evidence for this mechanism of control in *E. coli.*

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