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Non-physiological expression of UhpT does not lead to uncontrolled leakage of sugar phosphates out of *Escherichia coli* cells

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Abstract

De-regulated expression of *uhpT* under control of the *tac* promoter, by increasing concentrations of isopropyl-thio-β-d-galactoside, progressively inhibited the growth rate of *Escherichia coli* cells, to such an extent that growth was fully inhibited at 1 mM of the inducer. Significantly, addition of glucose 6-phosphate to the growth medium of the cells did not protect against this inhibition. Furthermore, efflux of sugar phosphates from the cells did not take place under these conditions, unless protonophoric uncouplers were added. We therefore conclude that the regulation of *uhpT* expression, i.e. via extracellular induction through a two-component system, did not evolve in order to prevent loss of essential metabolites from the cytoplasm under conditions when extracellular sugar phosphates are not available.

Keywords: Hexose-phosphate/phosphate antiporter; Glucose 6-phosphate; Two-component regulation; Extracellular induction; *uhpT*; Leakage of metabolites; *Escherichia coli*

1. Introduction

Microorganisms are forced to accurately adapt to the constantly fluctuating physical conditions in the extracellular environment. To achieve this, they are equipped with a large array of regulatory mechanisms, to express those genes that are required under a particular set of physical parameters of the extracellular environment.

These regulatory mechanisms are also recogniz-

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transport system. The archetype of regulation of the expression of a bacterial transporter is the lacIZYA system of *Escherichia coli* [3,4].

Quite a distinct mechanism of regulation of the expression of a number of secondary solute transporters has been revealed with the *uhp* system: of several transporters that allow bacteria to take up essential metabolites like hexose phosphates, triose phosphates and (di)carboxylic acids, the regulation of expression takes place via a so-called two-component regulatory system (TCRS; for reviews, see [5,6]). In this type of regulation, a sensor and regulator pair mediate a phosphorylation cascade that governs expression of the transporter via the phosphorylation level of the regulator at the expense of ATP. The best characterized system in this class is Uhp (for uptake of hexose phosphate; [7–9]). A striking characteristic of this form of regulation is that induction takes place only when a substrate (specifically glucose 6-phosphate) is present in the extracellular environment. This has been documented extensively for the Uhp system [10–12].

This type of regulation via a TCRS demands a continuous input of free energy in the form of ATP hydrolysis in the TCRS signalling system, apart from the synthesis of the proteinaceous components involved. The question therefore arises what the rationale is for this form of regulation of expression to evolve for this class of solute transporters, as an alternative to other regulatory mechanisms, like for instance the one described for the *lac* operon.

A common characteristic of the transporters regulated by a TCRS is the fact that the substrates which they recognize are also essential metabolites of intermediary metabolism. Therefore, it could be argued that it is important for the cells to prevent loss of these metabolites from the cytoplasm, via reversal of the uptake reaction, under conditions in which there is no extracellular supply of these metabolites. We have tested this proposal via bypassing the physiological regulation of expression of *uhpT*, by making use of a fusion construct in which *uhpT* is controlled by the *tac* promoter. This allows control of the expression of UhpT by varying the concentration of isopropyl-thio-β-D-galactoside (IPTG). The effects of non-physiological synthesis of UhpT on the growth rate of the cells and on the efflux of sugar phosphates out of the cells has been investigated.

### 2. Materials and methods

#### 2.1. Strains and plasmids

The following strains and plasmids were used in this investigation: *E. coli* JM109 (thi Δ(lac–proAB) endA1 gyrA96 relA1 supE44 recA λ−) and *E. coli* RK5000 (Δ(ileB–uhpABCT')) 2056 recA56, [13], R.J. Kadner, personal communication). The latter strain was a gift from Dr. R.J. Kadner, University of Virginia, Charlottesville, VA.

Plasmid pMI1 is a derivative of pTTQ19 with a 2.3-kb insert containing *uhpT*, under control of the *tac* promoter. It carries the origin of replication of pBR322 and confers ampicillin resistance to its host. It was kindly provided by Dr. P.C. Maloney (Johns Hopkins University, Baltimore, MD).

#### 2.2. Growth of organisms

The *E. coli* strains were grown at 37°C in Spizizen's minimal medium (phosphate concentration 125 mM; [14]) containing 27 mM glucose, the necessary amino acids and for *E. coli* JM109/pMI1 25 μg ml⁻¹ ampicillin. In the experiments in which we studied the inhibition of growth by overexpression of UhpT, minimal medium with 14 mM glucose as a C-source was inoculated with an overnight culture, to an initial optical density at 600 nm (OD₆₀₀) of 0.1. 20 ml of this cell suspension were incubated in 100-ml Erlenmeyer flasks with increasing concentrations of IPTG. Growth of the cultures was followed by measuring OD₆₀₀ at regular intervals.

#### 2.3. Measurement of efflux of glucose 6-phosphate from intact cells

An overnight culture, grown in minimal medium, was washed with minimal salts buffer and resuspended in the same buffer to an OD₆₀₀ of 3.5 (i.e., approximately 1 mg dry weight per ml). 20-ml portions of these washed cells were incubated in 100-ml Erlenmeyer flasks, after the addition of 25 μg ml⁻¹ chloramphenicol and 14 mM glucose. In some experiments, 20 μM of the protonophoric uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added. After centrifugation, the amount of glucose 6-phosphate in the extracellular medium was
2.4. Molecular biological techniques

Standard molecular biological techniques were applied as described [15].

2.5. Immunological detection of UhpT

Polyclonal antiserum containing rabbit IgG antibodies against UhpT was kindly provided by Dr. P.C. Maloney. To increase its specificity, 4 µl of the antiserum were incubated with 40 µl of a sonicated cell suspension of RK5000 (uhp-). After 5-fold dilution in minimal salts, this sample was slowly mixed for 1 h at 4°C. Cell material was removed from the resulting suspension by centrifugation in an Eppendorf centrifuge.

3. Results

To investigate the physiological consequences of controlled overexpression of UhpT, E. coli JM109/pMI1 was grown in minimal Spizizen’s medium with glucose as the carbon and energy source, and the growth rate was measured as a function of increasing IPTG concentrations. In the absence of IPTG, E. coli JM109/pMI1 grew in Spizizen’s minimal medium with a specific growth rate of 0.45 h⁻¹. The results in Fig. 1 show that increased (over)expression of UhpT indeed led to increased inhibition of the maximal specific growth rate of this strain, at IPTG concentrations larger than 10⁻⁵ M. Concentrations of 10⁻³ M IPTG or higher even gave rise to total cessation of growth, although in most cases growth resumed after overnight incubation. Restriction analysis of plasmid DNA, isolated from such cells, repeatedly showed that deletions in plasmid pMI1 occurred during incubation in the presence of high concentrations of IPTG (data not shown). It was observed that both vector and insert parts could decrease in size, indicating that the region around the tac promoter was prone to deletion under these circumstances. In agreement with this, it was observed that these cells became insensitive to growth inhibition by IPTG.

Several different interpretations at the molecular level can be forwarded to explain the IPTG-induced inhibition of growth. One possibility is that growth was retarded due to uncontrolled efflux of hexose phosphates from the cytoplasm of the cells, due to the steep concentration gradient of sugar phosphates across the cytoplasmic membrane (see also Introduction). We therefore included glucose 6-phosphate in the growth medium of the cells to test whether this could relieve the inhibition of growth by increasing concentrations of IPTG (Fig. 1, broken line). It was observed that inhibition of growth by IPTG under these conditions was indistinguishable from the incubation in the absence of glucose 6-phosphate. Similar results were obtained when it was tested whether fructose 6-phosphate could prevent inhibition of the rate of growth of the cells by the putative leakage of hexose phosphates from the cells (data not shown).

To investigate whether high levels of UhpT were indeed formed at IPTG concentrations lower than 1 mM, the membrane fraction of JM109/pMI1, with and without prior induction with 10⁻⁴ M IPTG, was analysed on Western blots, using specific antibodies against UhpT. The UhpT content of these membranes was compared with that of E. coli JM109 and E. coli RK5000, both induced with glucose 6-phos-
Fig. 2. Western blot analysis of membrane proteins of *Escherichia coli* JM109/pMI1, JM109 and RK5000, using a specific polyclonal antiserum directed against UhpT. Cells were grown overnight in minimal medium and a sample was taken of all strains to probe the uninduced cells. After the addition of glucose (7 mM) and the inducer (10^{-4} M IPTG for the *E. coli* strain JM109/pMI1 and 5×10^{-4} M glucose 6-phosphate for JM109 and RK5000), cells were incubated at 37°C for an additional hour, to allow for UhpT expression. Cells were diluted to an optical density at 600 nm of 1.0 and were sonicated. Membranes were collected via ultracentrifugation (50000×g; 1.5 h) and dissolved in Spizizen's minimal salts. Antiserum was pre-absorbed as described in Materials and methods. Lane 1, LMW markers; lane 2: JM109/pMI1 (not induced); lane 3, JM109/pMI1 (induced with IPTG); lane 4, JM109 (induced with glucose 6-phosphate); lane 5, RK5000 (induced with glucose 6-phosphate). The band corresponding with the UhpT protein is indicated by an arrow.

The results (Fig. 2) show that JM109/pMI1 produced a significantly higher amount of UhpT when compared with JM109, even when the latter strain was induced with saturating concentrations of glucose 6-phosphate. This amount of UhpT was just sufficient to show a faint cross-reaction with the antibodies. The difference between induced and uninduced cells of JM109/pMI1 clearly reveals the high amount of UhpT present in the membrane of JM109/pMI1 cells after induction with 10^{-4} M IPTG. The increased synthesis of UhpT in *E. coli* JM109, after introduction of plasmid pMI1, leads to the conclusion that upon growth of the cells in glucose minimal medium, due to its presence in multi-copy form, the tac promoter shows considerable activity already without added IPTG.

To determine whether any glucose 6-phosphate leaked out of the cells into the external medium upon de-regulated expression of UhpT, in the absence of added extracellular glucose 6-phosphate, the concentration of this metabolite was measured in the supernatant of cultures of JM109/pMI1, with and without induction with IPTG (Fig. 3). Only low amounts of glucose 6-phosphate (up to about 30 μM) could be detected without significant differences between induced and uninduced cells. The difference in external glucose 6-phosphate between JM109 and JM109/pMI1 is probably caused by the high amounts of UhpT present in JM109/pMI1.

Because of these relatively low glucose 6-phosphate concentrations, next we tested whether any leakage of glucose 6-phosphate through UhpT into the extracellular medium can occur during metabolism of added glucose. To avoid interference by protein synthesis in these experiments, cells were incubated with 14 mM glucose in the presence of 20 μg ml^{-1} chloramphenicol. Extracellular concentrations of glucose 6-phosphate were measured in the supernatant of cultures of JM109 without and with pMI1 (see Fig. 4A and B, respectively). In cultures of JM109 and RK5000 (carrying an uhpT deletion; data not shown), again only very low amounts of glucose 6-phosphate in the range of 10 μM could be detected extracellularly. Prior induction with glucose 6-phosphate of UhpT gave rise to an increase in the extracellular glucose 6-phosphate concentration in
Fig. 4. The effect of CCCP on the efflux of glucose 6-phosphate from cells incubated in minimal medium. Cells were grown in minimal medium with glucose as carbon source and washed and incubated (in the presence of chloramphenicol, 25 μg ml⁻¹), in minimal salts medium. Glucose (14 mM) was added at the start of the experiment. In some of the incubations 20 μM CCCP was included. The amount of glucose 6-phosphate was assayed as described in Materials and methods. (A) JM109: (■) not induced, no CCCP added; (▲) induced with glucose 6-phosphate (5 × 10⁻⁴ M), no CCCP added; (●) not induced, CCCP added (20 μM); (○) induced with glucose 6-phosphate (5 × 10⁻⁴ M), CCCP added (20 μM). (B) JM109/pMI1: (■) not induced, no CCCP added; (▲) induced with IPTG (10⁻⁴ M), no CCCP added; (●) not induced, CCCP added (20 μM); (○) induced with IPTG (10⁻⁴ M), CCCP added (20 μM).

The decrease in external glucose 6-phosphate concentration, starting between 2 and 3 h after initiation of glucose metabolism, is caused by catabolism of glucose 6-phosphate. Simultaneous measurements showed that by this time the extracellular glucose had almost completely been consumed (data not shown).

Efflux from the cells was only found when the energy metabolism was interfered with by the addition of CCCP. This uncoupler also impaired the glucose metabolism, a significant decrease of the rate of glucose catabolism was observed in its presence (data not shown).

4. Discussion

To investigate whether the strict on/off regulation of the transcription of the uhpT gene is necessary to prevent unwanted loss of metabolites, the effect of deregulated synthesis of UhpT on the maximal growth rate and extracellular glucose 6-phosphate concentrations was determined. It was observed that uncontrolled synthesis of UhpT gave rise to a severe impairment of growth. However, this did not appear to be the result of excessive loss of phosphorylated sugars from the cells. Rather, it appeared more likely that this growth inhibition was due to overcrowding of the export machinery of the cells (i.e. the Sec system; [16]) or the cytoplasmic membrane itself.

As shown in Fig. 4, in an incubation under physiological conditions no significantly greater amount of glucose 6-phosphate could be found in the culture medium of *E. coli* JM109/pMI1, even after non-physiological overexpression of UhpT, as compared to a culture of a strain that did not carry the pMI1 plasmid. Significant leakage of glucose 6-phosphate from intermediary metabolism to the extracellular environment is only observed when energy metabolism was interfered with, for instance by the addition of CCCP (or valinomycin and/or nigericin; data not shown). The lack of efflux from the energetically unperturbed cell is difficult to explain, keeping in mind the generally accepted notion that glucose 6-phosphate/phosphate exchange by UhpT is electroneutral [17]. One explanation for this observation...
could be that an inwardly directed phosphate gradient was present that prevented sugar phosphate leaking out of the cells. The explanation for the effect of uncouplers might then be at the level of the magnitude of the intracellular inorganic phosphate concentration. Alternatively, the protonophore may also exert its effect via disturbance of for instance the intracellular pH. The interpretation of these latter experiments, however, is hampered by the inhibition of the added CCCP on glucose catabolism, indicated by the severely diminished rate of catabolism of glucose in the presence of CCCP.

No straight-forward explanation can yet be provided for the observation that a number of solute transport systems for essential intermediary metabolites are (tightly) regulated through a two-component system, with extracellular induction. One may hypothesise that, since the transcription of \textit{uhpT} is regulated by glucose 6-phosphate, an essential metabolite, an intracellular regulation as used in the \textit{lac} operon is not feasible, because of the continuous intracellular presence of the inducer at variable concentrations.

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References