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Yeast cultures with UCP1 uncoupling activity as a heating device

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Uncoupling proteins (UCPs) are mitochondrial transporters that facilitate controlled dissipation of the proton gradient and thus regulate energetic efficiency. The heat generating capacity of UCP from brown adipose tissue was investigated in yeasts expressing the protein recombinantly under conditions in which the temperature of the growth medium was measured directly. A Liquid Culture Calorimeter (LCC) was built consisting of a thermally isolated culture flask able to keep yeast cultures warm without resorting to additional heating. The exact internal temperature of the cultures was monitored for 24 h through a thermocouple connected to a data logger. Under these conditions, significant temperature increases (1 °C) in the media were recorded when yeast strains expressing endogenously active UCP1 mutants were grown. This is the first direct evidence, in a eukaryotic microbial model, of a temperature rise associated with uncoupling activity, and could be seen as the first step toward developing a biological heating device.

Introduction

Living systems operate under the principle of energetic efficiency. Metabolic networks are interrelated to ensure that energy usage matches cell requirements. Thus, mitochondrial oxidative phosphorylation couples the rate of substrate oxidation in the respiratory chain to the cellular ATP demand. This coupling takes place through a proton gradient, generated across the inner membrane by the respiratory chain, which is used by the H+-ATPase to drive ATP synthesis. However, there is a wide variety of energy dissipatory mechanisms that bypass the constraints imposed by mitochondrial respiratory control. These energy-spilling processes fulfill many different physiological functions such as eliminating excess calories, maintaining body temperature or controlling the production of reactive oxygen species.

Mitochondrial substrate oxidation can be increased by mechanisms that utilize ATP. For example, shivering is an acute response to cold stress where activation of ATPases in the muscles increases the rate of energy utilization while metabolic activity does not lead to the coordinated contractions required to perform mechanical work. There are also mechanisms that activate futile cycles leading to high rates of ATP hydrolysis. One example is the simultaneous functioning of phosphofrutokinase and fructose1,6-bisphosphatase in the thoracic muscles of the bumblebee that constitutes a potent heat generating biological device [1]. Other thermogenic mechanisms, mainly exploited by plants and eukaryotic microorganisms, involve the dissociation of electron transfer in the respiratory chain from proton pumping with the concomitant

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decrease in energy conservation. Thus, the induction of a cyanide-insensitive alternative oxidase, which transfers electrons from ubiquinone to oxygen without proton pumping, is the thermogenic mechanism of flowering *Araceae*. These plants attract insects by considerably raising the temperature of their spadices to volatilize malodorous compounds [2].

UCPs are mitochondrial transporters that catalyze the reentry to the matrix of the protons that have been pumped out by the respiratory chain. This dissipatory mechanism operates, for example, in mammalian brown adipose tissue. This thermogenic organ is activated at birth, on awakening from hibernation, by chronic cold-exposure and, at least in rodents, to burn excess ingested calories. The activity of the uncoupling protein from brown adipose tissue, UCP1, is tightly regulated: cytosolic purine nucleotides keep the protein inhibited while fatty acids are the physiological activators [3]. In mammals there are four other genes encoding proteins considered to be UCPs [4]. While UCP1 is only present in brown adipose tissue, UCP2 is ubiquitous and UCP3 is expressed in skeletal muscle and brown adipose tissue. UCP4 and BMCP1 (UCP5) are predominantly expressed in the nervous system. Genome sequencing has revealed that UCP distribution is widespread, since they are to be found not only in all phyla of the animal kingdom but also in plants and fungi. It must be stressed, however, that neither the physiological roles of most members of the UCP family nor the regulation of their activity are fully understood [5,6].

The work presented here aims to exploit the thermogenic properties of the UCP1 from brown adipose tissue in order to develop a heat-producing system in yeast. This can be considered as the first step toward a biological heat-donor device with a variety of industrial applications, such as sustaining microbial growth. Additionally, this system could be used as an energy-spilling device that could increase metabolite production by industrial microorganisms.

Materials and methods
Strains and plasmids
Four genetically engineered *Saccharomyces cerevisiae* strains were used: UCP1, Control, Gly175Δ and Gly76Δ. Strains were obtained by transforming strain W303 (a/a; ade2-1O; his3-11,15; leu2-3,112; ura3-1; canl-100; trp-) with the pYeDP-1/8-10 vector [7] containing the coding sequences of mammalian (rat) UCP1 gene under the control of the *gal-cyc* promoter [8]. Control yeasts were transformed with the same vector but with the sequence of UCP1 inserted in antisense orientation. Gly175Δ and Gly76Δ mutant strains had deletions on Gly175 and Gly76 of the UCP1 sequence, as previously described [9].

Liquid Culture Calorimeters
In order to provide maximum thermal insulation to the liquid cultures and to allow real-time recording of the inner temperature, Liquid Culture Calorimeters (LCCs), rather than standard Erlenmeyers flasks, were used. LCCs were constructed as follows: one-liter commercial Valira (Reus, Spain) vacuum thermo flasks (mod. 6128/52) were covered with an extra insulation layer (Armaflex© foam). Plastic lids were then drilled and a T thermocouple (Cu-Constantan) inserted through the hole to reach the bottom of the flask. The gap between the thermocouple and the lid was sealed with Parafilm (Figure 1A).
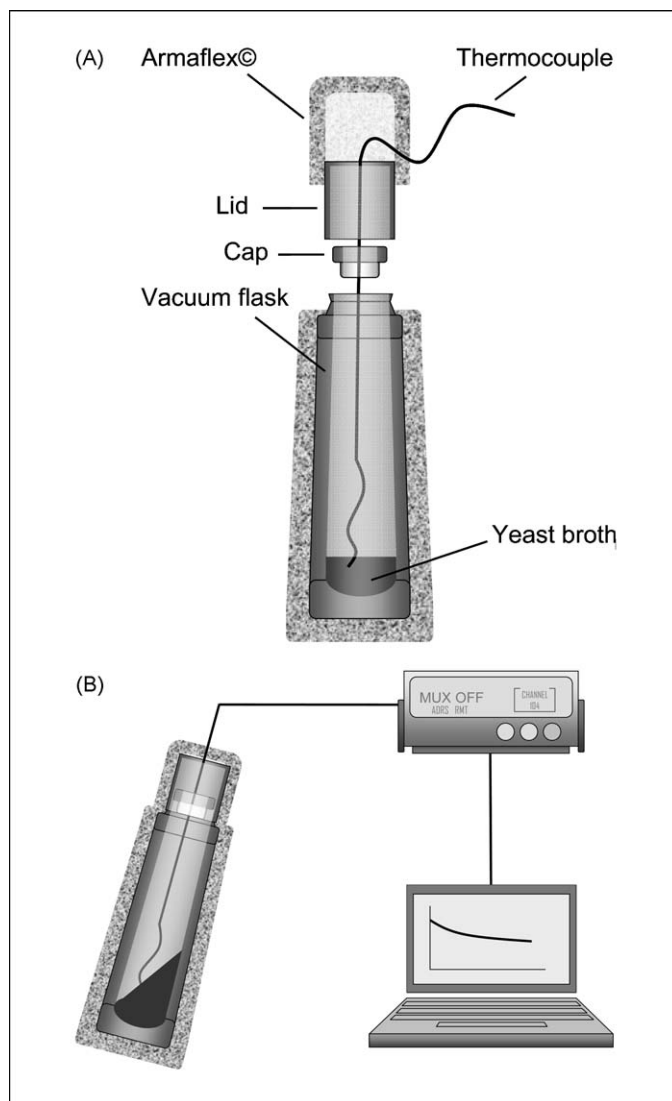
FIGURE 1

(A) Liquid Culture Calorimeter (LCC) design and (B) LCC internal temperature recording system through a thermocouple connected to a data logger and a PC.

The internal temperature of the LCCs was recorded by a thermocouple connected to a PC through a data logger as shown in Figure 1B. Thermocouples were connected to a voltage acquisition card inserted on the data logger and the data logger was connected via an RS-232 cable to a PC with specifically designed acquisition software based on Vee Pro. This system enabled measurement frequency to be set and temperature readings to be stored on the computer. The thermocouples were calibrated according to standard thermoresistance, and the precision of the system was calculated to be as small as 0.01 °C.

For calibration purposes, all LCCs were loaded with warm (32 °C) water, subjected to shaking (250 rpm) and the changes in internal temperature were recorded for 20 h with the system set as described above.

Culture conditions
Yeast strains were cultured on solid SD medium (0.67% (w:vol) yeast nitrogen base, 2% glucose, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine and 2% agar). After 48 h of incuba-

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tion at 28 °C, a single colony was used to inoculate 30 ml of liquid SD medium into 100 ml Erlenmeyers. These precultures were grown at 28 °C and 180 rpm overnight.

LCCs were sterilized by successive rinsing with bleach (10% vol:vol), sterile boiling water and finally with ethanol, and they were then filled with 100 ml of warm (31 °C) liquid SP medium (0.67% yeast nitrogen Base, 0.1 % potassium phosphate, 0.05% glucose, 2% lactic acid, 0.1% casamino acids, 20 mg/l tryptophan and 40 mg/l adenine) at an initial OD_{600} of 0.2–0.6 with overnight SP cultures prepared as described above. LCCs were incubated with shaking (250–275 rpm) on a Certomat MoII orbital shaker (Sartorius, Goettingen, Germany) without a lid at room temperature. Induction of the *gal-cyc* promoter was performed adding sterile galactose to yield a final 1% (w:vol) concentration. The internal temperature of the growing cultures was monitored as described (Figure 1B) and recorded on the PC for 15–50 h. At the end of the experiments, aliquots were taken and subjected to microscopic observation in order to discard bacterial contamination. For

growth kinetics, one-liter Erlenmeyers containing 100 ml SP liquid medium set at an initial OD_{600} of 0.2–0.6 from the overnight yeast cultures were shaken at 180 rpm on a standard orbital shaker set at 28 °C for 10 h. Aliquots were taken every 90 min and OD_{600} was measured with an 6131 Eppendorf BioPhotometer.
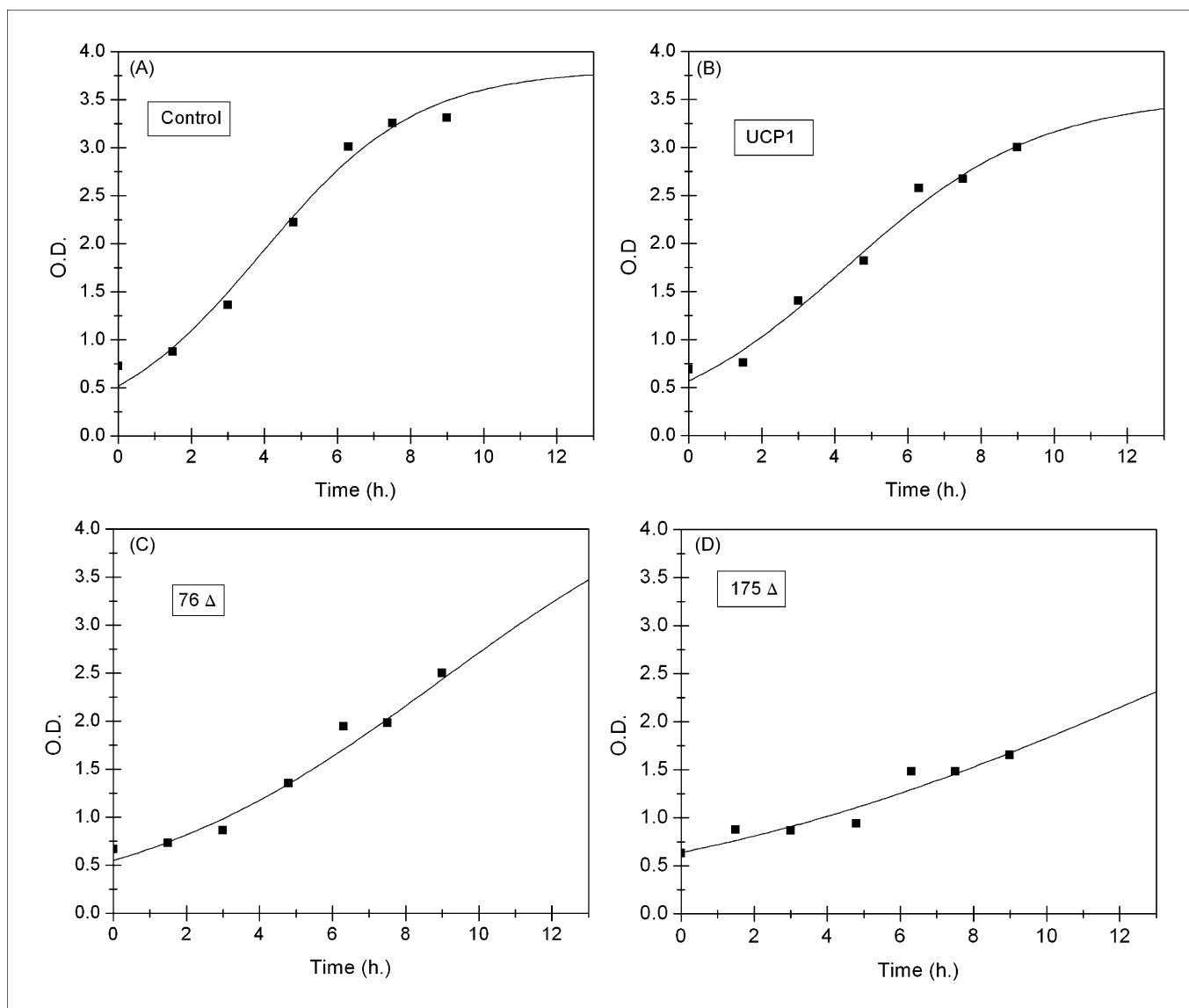
Modeling
Four equations were used to represent a UCP-based heat generating system:

$$\frac{dN}{dt} = Nr\left(1 - \frac{N}{K}\right) \tag{1}$$

$$\frac{dG}{dt} = -\beta_1 G \tag{2}$$

$$\frac{dH}{dt} = \alpha \frac{(G/K_1)^n}{1 + (G/K_1)^n} - \beta_2 H + \gamma \tag{3}$$

FIGURE 2

Growth kinetics characterization curves for the four yeast strains: UCP1 (A), Control (B), 175Δ (C) and 76Δ (D) as determined by OD_{600} measures of the cultures taken every 1.5 h.

$$\frac{dT}{dt} = -k(T - T_a) + \xi H \qquad (4)$$

A black-box model was built to simulate the temperature changes of the system as a function of growth rate, galactose concentration and UCP expression. Eq. (1) represents culture growth (logistic growth of the four strains studied); Eq. (2) represents the variation in galactose concentration (galactose being the *gal-cyc* promoter activator); Eq. (3) aims to reproduce the variation in the concentration of UCP (the first term represents the UCP expression promoted by galactose, the second term UCP degradation, and the third one the basal production rate); finally Eq. (4) represents the temperature changes (the first term represents heat loss from the calorimeter to the environment (according to Newton's law) and the second represents temperature increase as a consequence of UCP activity.

Results

Growth kinetics of recombinant yeast strains
Since UCP1 is kept inhibited by cytosolic purine nucleotides, its thermogenic activity requires the presence of an activator, namely fatty acids or retinoids. However, we have previously characterized a series of UCP1 mutants with modified regulatory properties [9,10]. Thus, the deletion mutants Gly76Δ and Gly175Δ, which are not inhibited by purine nucleotides and can catalyze proton transport even in the absence of activating compounds, were chosen as the best candidates for the present project, since they represent the simplest endogenously active UCP-based dissipatory system.

Growth rate of the four yeast strains was studied by monitoring OD_{600} of the growing cultures. Typical growth kinetics are shown in Figure 2. Both reference strains (UCP1 and Control) exhibited a similar growth rate, with a maximal OD_{600} at the end of the experiment of about 3.5 (Figure 2A,B, respectively). Both curves were not significantly different, indicating that growth kinetics of UCP1 and Control strains, neither of which express functional UCP1, are very similar. By contrast, mutant strains Gly175Δ and Gly76Δ exhibited a marked delay in growth compared to Control strains, with a maximal OD_{600} of only 2.5 after 10 h. Among mutant strains, the kinetics of Gly175Δ differed significantly from that of Gly76Δ, with Gly175Δ growing more slowly that Gly76Δ (Figure 2C,D).

The experimental results were fitted to Eq. (1) in order to obtain parameters r and K for the four strains. The values of these parameters are shown in Table 1. The lower growth rate of the mutants observed in the figures is reflected in the values of parameter r (which is related to the slope of the growth curves); this parameter is lower for the strains with active UCP.
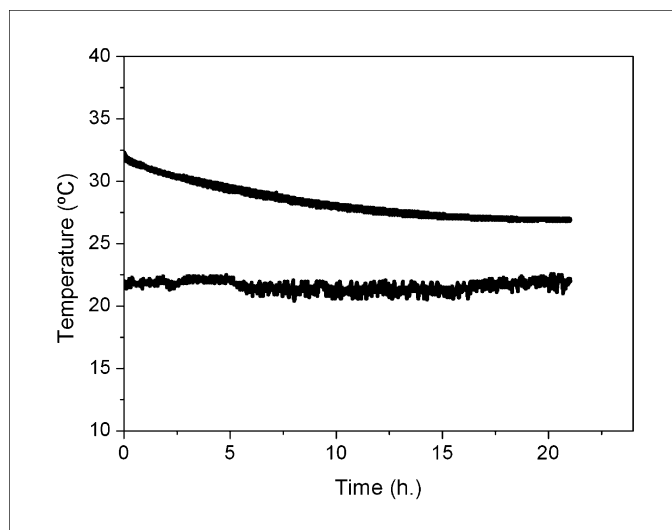
FIGURE 3

Calibration of LCCs: temperature changes of Liquid Culture Calorimeters (LCCs) containing warm (32 °C) water and shaken under the conditions described in Materials and Methods. The lower curve shows room temperature.

LCC characterization
Assuming that the specific heat of SP medium is approximately the same as that of water, LCC heat losses were characterized with pre-warmed sterile water under the same conditions as those used for yeast growth. Temperature changes inside the LCCs, under shaking, were recorded for 24 h and are shown in Figure 3.

Changes in the inner temperature of the four devices revealed that all four LCCs behaved very similarly in terms of heat loss. This indicates that heat loss was the same for the four LCCs. This loss was characterized by determining a value for the parameter k of Eq. (4) of 0.01313 ± 0.00057.

Temperature kinetics of the yeast strains
The internal temperature of the four yeast cultures growing inside the LCCs was measured as described in Materials and methods. Preliminary experiments were conducted with at an initial OD_{600} of 0.2 taken from an overnight SP culture and under moderated (250 rpm) shaking. Under these conditions, significant temperature increases were only observed after very long ($\geq$24 h) incubation times (data not shown). Since long incubation times in sealed containers, like the LCC used here, might result in insufficient oxygen for respiration processes to be detected, culture conditions were changed to obtain maximal aeration. Shaking was increased to 275 rpm and the LCCs were set at a slight (10°) tilt. Additionally, the initial OD_{600} of cultures was increased to 0.2. Culture temperatures were recorded for 12–24 h under these conditions and are shown in Figure 4. The temperature of all the strains dropped about 1 °C in the first hour, from an initial 30–30.5 °C down to 29–29.5 °C. The temperature of two strains, UCP1 and Control, progressively decreased to around 28–28.5 °C during the rest of the experiment.

By contrast, changes in the temperature of the mutant strains Gly76Δ and Gly175Δ showed a markedly different pattern, with a significant increase in the temperature of the cultures compared to UCP1 and Control strains (Figure 4). The internal temperature of
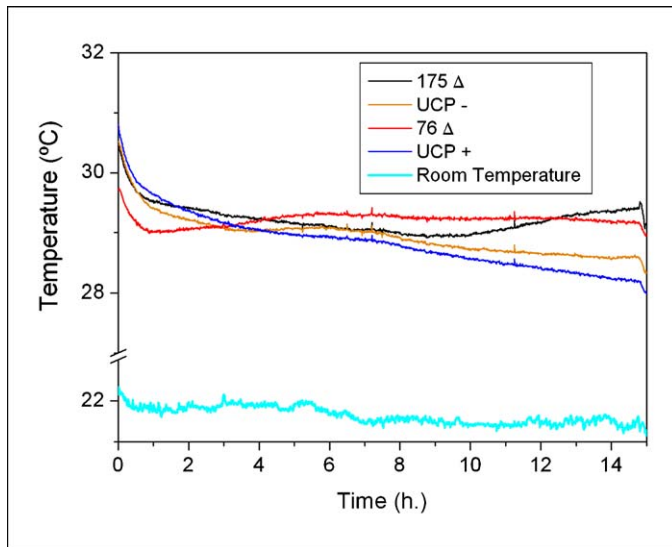
TABLE 1

Growth parameters of the four tested strains calculated according to Eq. (1) (see text for details). Standard deviations are shown

	Control	UCP1	Gly76Δ	Gly175Δ
K	3.7 ± 0.3	3.5 ± 0.4	5 ± 3	4.3 ± 1.1
R	0.47 ± 0.8	0.38 ± 0.07	0.23 ± 0.07	0.14 ± 0.06
N₀	0.52 ± 0.11	0.57 ± 0.09	0.55 ± 0.09	0.64 ± 0.11

FIGURE 4

Temperature change curves for the four strains in the LCCs under the experimental conditions described in Materials and methods showing temperature increases of mutant strains 175Δ and 76Δ. These strains have uncoupling activity even in the absence of activating ligands. The lower curve shows room temperature.

the LCC containing strain Gly76Δ rapidly increased after the first 60 min and reached a maximum after about 5 h of incubation. From this time onwards, the culture slightly decreased in temperature. The culture with strain Gly175Δ also increased in temperature compared to UCP1 and Control strains, but with a significant delay compared to Gly76Δ. In fact, Gly175Δ started heating up after 8–10 h of incubation and reached a maximum (about 29.5 °C) after about 15 h. At the end of 15-h experiments, internal temperatures of mutant strain cultures were typically about 1 °C hotter than those of UCP1 strains and the Control.

Using the data obtained from Figure 4, in which culture temperature was registered as a function of time, parameters β_1, α, β_2, γ, K_1, n and ξ of Eqs. (2)–(4) were calculated from a minimal square fitting algorithm with Simulink© software, with parameters α, β_2, γ, K_1, and n representing the expression of UCP. Since all strains share the same promoter, we assumed that the value of parameters of Eq. (3) should be the same for the four strains. Parameters β_1 and ξ were considered as independent for each strain because different UCP sequences might result in different uncoupling activities.

Table 2 shows the value of the parameters associated with UCP expression. According to the model, it can be concluded that UCP has quite a long mean lifespan (approximately 30 h) and that the basal UCP expression (without galactose) is negligible. The parameter n is shown without error because this parameter was con-

TABLE 2

UCP1 expression parameters. Standard deviations are shown

Parameter	Expression
A	6.9 ± 0.4
K_1	0.15 ± 0.08
β_2	0.034 ± 0.006
Γ	0.012 ± 0.003
N	2

TABLE 3

Heating effect for mutant strains calculated according to Eq. (1)

	Control	Gly76Δ	Gly175Δ
ξ	0	3.14 ± 0.89	3.7 ± 0.3

sidered constant and with a value of two in order to reduce the solutions of the space parameter.

The thermal effect exerted by UCP on the culture (ξ) was calculated (Table 3). The value obtained for Gly175Δ was slightly higher than that obtained for Gly76Δ. This is in concordance with the growth curves, and it should be noted that a value of zero for the Control strain is in concordance with its temperature changes. Figure 5 represents the typical fits obtained

Discussion

UCPs are members of a protein superfamily comprising the metabolite transporters of the mitochondrial inner membrane [11]. These mitochondrial carriers are known to switch from their specific carrier mechanism to a channel/pore mode. The altered carried function is probably of pathophysiological significance [12]. We have previously reported that a number of modifications induced either chemically or by site-directed mutagenesis can alter both the substrate specificity and protein activity regulation [9,10,12]. This knowledge was used to select UCP1 mutants with the properties required to construct new microorganisms able to generate heat. We have previously reported that when UCP1 is recombinantly expressed in Saccharomyces cerevisiae it retains all its functional properties [7]. When expression is induced by galactose, the Control and UCP1 strains do not vary significantly in growth rate owing to UCP1 inhibition by the cytosolic nucleotides. However, mutant strains Gly76Δ and Gly175Δ decrease significantly in growth rate owing to the endogenous activity of the mutant proteins [9]. The results reported here show that, under appropriate conditions (thermal isolation, high aeration and exact real-time temperature measurement) the two mutant strains exhibiting delayed growth were found to significantly heat their culture broth. Although previous studies have reported heat production measured by a microcalorimeter assay [13], this is the first time UCP1 uncoupling activity has been demonstrated to produce a measurable increase in temperature. This increase could be recorded thanks to the thermally isolated culture instrument (Liquid Culture Calorimeter). This simple recording system might be useful in other real-time experiments with microbial cultures that need a thermally closed environment in order to detect small variations in the internal temperature of the cultures.

Besides confirming UCPs' heating potential, our aim was to implement a biological UCP-based device with biotechnological application, either as a heat producer or as a dissipatory mechanism. The former application encompasses the use of recombinant yeast as a source of energy thereby dispensing with external heating, needed for microbial growth, as well as implementing it as a heat donor. In theory, a UCP1-expressing yeast culture could be thermally coupled and transfer its heat to another biological or chemical reaction, including another microbial culture.

However, probably the most interesting application of a UCP1-based device would be a general dissipatory system that could be
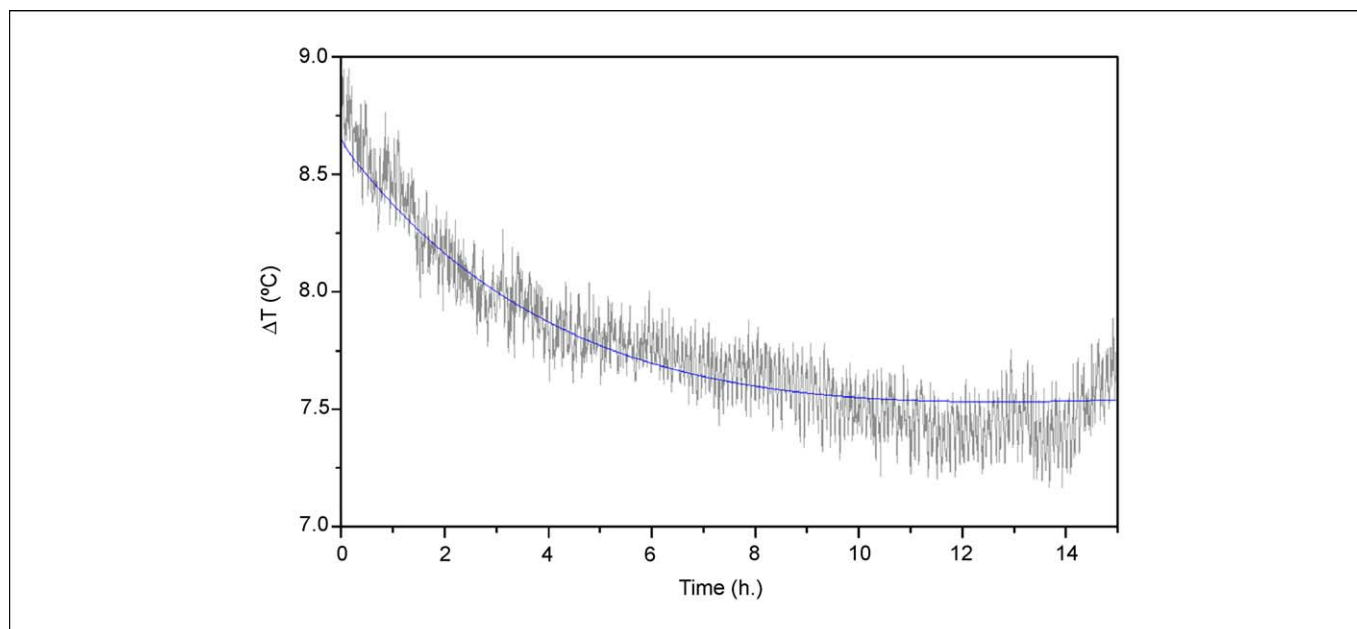
FIGURE 5

Graphic representation of the goodness of fit of the temperature reached. ΔT represents the difference between the environment and the inlet of the calorimeter.

used with genetically engineered microorganisms. GM microorganisms are often used in biotechnology as a source of metabolites, but large-scale industrial production of secondary metabolites requires large flows of carbon and energy. Since the energetic and redox balance must be maintained, excess energy in the form of reducing equivalents must be dissipated. When microorganisms are grown under aerobic conditions, one possibility is to facilitate the re-oxidation of the coenzymes in the respiratory chain. This requires the induction of pathways to lower oxidative phosphorylation efficiency and thus accelerate respiration. Uncoupling agents, such as benzoic acid or dinitrophenol, have been used for this purpose in industrial applications [14,15]. Excess energy can also be detected in wild-type microorganisms, since the observed growth yield is known to decrease under substrate-sufficient conditions. This indicates that the excess substrate causes uncoupling between anabolism and catabolism, leading to the dissipation of non-growth energy [16]. This phenomenon has been called 'uncoupling', 'overflow metabolism' and 'energy spilling'. As a result, it appears that many microorganisms spill energy and the few that do not, exhibit rapidly decreasing viability [17]. Such spilling processes, which are necessary to optimize microbial performance and metabolite production, have not been addressed yet. Our results suggest that uncoupling proteins, such as UCP1, might be the base not only of simple cell heating devices but also of dissipatory devices integrated in genetically modified microorganisms to dissipate the excess energy associated to metabolite production.

Sequences

UCP1 as well as mutant sequences Gly76Δ and Gly175Δ bearing the appropriate Biobrick prefix and suffix are available in the M.I.T. Registry of Standard Biological Parts (http://partsregistry.org/Main_Page) as BBa_K141000, BBa_K141003 and BBa_K141002, respectively.

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