43 Hemin reconstitutes the growth of an H^+-ATPase negative mutant of *Lactococcus lactis*

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Introduction

The H^+-ATPase of *Lactococcus lactis* allows the generation of a proton motive force and the control of the internal pH by utilising ATP. The *atp* operon encoding the membrane bound H^+-ATPase of *L. lactis* was recently cloned and characterised in our group, and subsequent work produced a strain containing the H^+-ATPase operon under the control of an nisin-inducible promoter (NICE-system) [10]. Under non-induced conditions this strain, designated PJ4699, is H^+-ATPase-negative. It cannot grow on SA agar plates in the absence of nisin leading to the conclusion that the H^+-ATPase is vital for *L. lactis* [10].

ATP production in this organism is limited to glycolysis due to the lack of oxidative phosphorylation. In medium containing an excess of glucose, this purely catabolic pathway merely generates two moles of ATP per mole of glucose with lactate as the major end product. Finding another means to generate a proton gradient could result in higher biomass due to energy savings from proton transport.

It has been reported that the addition of hemin, an iron-protoporphyrin which is a prostethic group of cytochromes, to growth media, reconstituted a cytochrome-like NADH oxidase activity in some Streptococci species [1] when cells were grown aerobically. A ~25% increase in ATP yield on glucose, which was reflected by a higher final biomass, was observed in a *Streptococcus faecalis* (reclassified to *Enterococcus faecalis*) strain. The researchers concluded that *E. faecalis* could benefit from oxidative phosphorylation and demonstrated the existence of cytochromes under aerobic conditions with hemin [1]. This conclusion is supported by a report, which showed proton efflux in *in vitro* experiments when *E. faecalis* cells had been grown aerobically in the presence of hemin [8].

Although, *L. lactis* is somewhat related to *E. faecalis*, it is not generally accepted that *L. lactis* can benefit from the presence of oxygen. The differences between these two species are well documented and are reflected, for example, by the differences in end products under aerobic conditions. *L. lactis* predominantly produces lactate, whereas *E. faecalis* produces mainly acetate.

Here we report that by adding hemin to the *L. lactis* strain which has nisin dependent expression of H^+-ATPase, growth is reconstituted in the absence of nisin. The results are discussed in the context of possible induction of alternative H^+ transport systems by hemin.

Materials and methods

Bacterial strains and growth conditions will be described in details elsewhere (Blank *et al.*, manuscript in preparation). Briefly, the strains studied were *L. lactis* subsp. *cremoris* PJ4662 (MG1363 containing pAK80 [12]) and a strain having the *atp* operon under the control of a nisin-inducible promoter, designated PJ4699. pAK80 is an *E. coli*-*L. lactis* shuttle vector, including a *β-gal* reporter gene and an erythromycin resistance gene for selection. Cells were grown in SA medium, a chemically defined medium, comprising of 19 amino acids, trace elements and vitamins, optimised for these nutritionally fastidious bacteria [11]. The media was supplemented with 10 g/l glucose and 15 g/l agar when preparing plates. Growth experiments were performed at 28 °C with a 10 ml culture volume (20 ml test tubes) on an orbital shaker (New Brunswick Scientific, G25) at 240 rpm with a 60° angle to ensure full aeration. The media was supplemented with 5 μg/ml erythromycin. Hemin (Sigma, H-2250) was added to a final concentration of 5 μg/ml (plates) and 1 μg/ml (liquid culture). Nisin was added to a final concentration of 16 ng/ml. A previously reported hemin concentration of 20 μg/ml [2] was inhibitory in our experiments. Plates were incubated at 30 °C. Optical density was determined at 600 nm using a spectrophotometer (Shimadzu).

Measurements of growth rate and growth yield (yield of biomass/mole of glucose) were performed in liquid SA medium, supplemented with a limiting concentration of glucose as described previously [11].

Results and discussion

Hemin restored the growth of an H^+-ATPase mutant

In the *L. lactis* strain PJ4699, the promoter of the chromosomal *atp* operon has been replaced with the *nisA* promoter, and the expression of the H^+-ATPase then

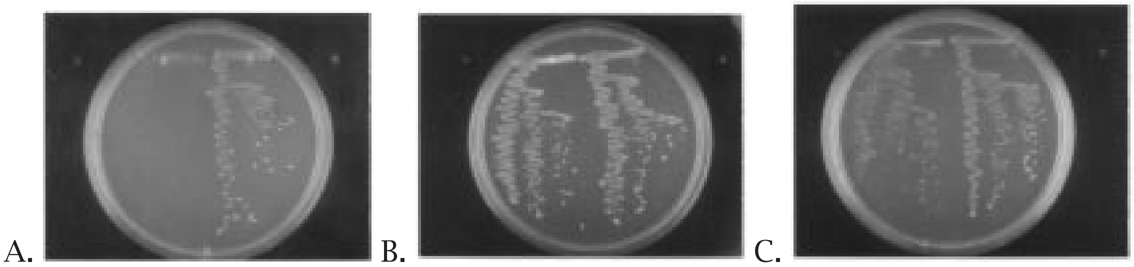

Fig. 43.1 Hemin effect on growth of L. lactis strains PJ4699 (left) and PJ4662 (right). A. SA agar plate; B. SA agar plate + 5 μg/ml hemin; C. SA agar plate + 16 ng/ml nisin.

only takes place in the presence of nisin, the inducer of the *nisA* promoter. Growth of PJ4699 is practically normal when nisin is added to the plate (Fig. 43.1C). In the absence of nisin, the cells becomes H^+-ATPase negative and growth on SA agar plates is abolished (Fig. 43.1A), which demonstrates that the H^+-ATPase is essential for growth of *L. lactis* under these conditions [10]. Note that the few colonies visible on the SA plate without nisin (Fig. 43.1A) are revertants, which regained the ability to express the *atp* operon.

Surprisingly, the addition of hemin to the SA plates completely restored the growth of this mutant strain (Fig. 43.1B), and it therefore appears that hemin replaces the function of the H^+-ATPase. It was also interesting to note that the colonies of the mutant strain was bigger on the hemin plate (Fig. 43.1B) than on the SA plates with hemin (Fig. 43.1C). These data indicate the presence of processes in the cells, which can be enhanced by the addition of hemin, and which control the growth of the wild type cells significantly. Growth is here used in the broad sense, since the growth of colonies on plates reflect (i) the growth rate of the cells, (ii) the efficiency by which they use the substrates provided (yield) and (iii) the extent to which they are inhibited by their own excreted by-products.

When we compared the growth of the mutant and the reference strain on hemin and hemin+nisin plates, there was no difference for either of the strains (data not shown). This result indicates that, once hemin is present, the H^+-ATPase has insignificant control on the growth of these cells.

Does the H^+-ATPase negative mutant require a proton gradient?

It is generally believed that a proton gradient is vital for the cell to maintain transport processes and a previous report showed that a *L. lactis* strain with reduced membrane-bound H^+-ATPase was defective with respect to controlling its internal pH [3]. To test whether a proton gradient was actually restored by hemin in the H^+-ATPase negative mutant, we added the uncoupling agent 2,4-dinitrophenol (DNP) to hemin plates. At a DNP concentration of 10 mM growth of strain PJ4699 was abolished, which shows that a proton gradient is indeed important for the growth of these cells, and indicates that a reconstituted proton gradient in PJ4699 (via hemin) is eliminated by the use of the uncoupler.

Interestingly, the wild type reference strain PJ4662 still grew in the presence of 10mM DNP, even though the growth rate was significantly reduced. This result demonstrates the requirement of the H^+-ATPase for growth under these conditions.

Do lactococci have cytochromes?

The reconstitution of growth of the H^+-ATPase *L. lactis* strain PJ4699 on plates with hemin, immediately raises the question, whether hemin complements a proton transport system other than the H^+-ATPase, and if so, which system is it. Earlier experiments done with *Enterococcus faecalis* [2], showed that cytochromes could be detected after hemin had been added to aerobic cultures, and this may also be true for *L. lactis*. Indeed, the genome-sequencing project of *L. lactis* subsp. *lactis* strain IL1403, a strain that is closely related to our model organism, MG1363, has revealed the presence of a cytochrome gene in the genome [9]. Recently (1999, 6th Symposium on Lactic Acid Bacteria, Veldhoven, The Netherlands) P. Duwat *et al.*, reported that a cytochrome deletion mutant of *L. lactis* subsp. *cremoris* MG1363 lost the hemin phenotype, directly confirming that hemin is complementing a functional cytochrome.

Can end product efflux account for the generation of a proton gradient in the H^+-ATPase negative mutant?

In the literature there have been many reports on alternatives to the H^+-ATPase and cytochromes for proton gradient generation in bacteria, e.g., end-product efflux [4] and malolactic fermentation [5,6].

The group of Konings showed that end product efflux can generate a proton gradient by a symport mechanism (for review, see [14]). The stoichiometry in mol protons/mol end product of this transport is highly depending on the end product gradient and can vary from 0.9 (a net import of protons) to 2.0. Lactate efflux can not generate a proton gradient sufficient for growth, since PJ4699 does not grow in the absence of nisin (Fig. 43.1A). However, the addition of hemin might reconstitute an NADH-oxidase activity (a redox sink) that would lead to a switch in metabolism from lactate to acetate as previously observed [13]. The efflux of acetate could then result in the generation of a proton gradient.

To test this hypothesis, we grew the H^+-ATPase negative mutant, PJ4699, on a series of plates containing hemin and increasing concentrations of acetate (Table 43.1).

However, the growth of strain PJ4699 was not abolished under the tested conditions, even when the acetate concentration reached 200 mM. Under these condi-

Table 43.1 Growth of strains on plates with varying acetate concentrations. A. Incubation for 40 hours at 30 °C. B. Incubation for 64 hours at 30 °C.

A.

	15 mM Acetate			50 mM Acetate			100 mM Acetate			200 mM Acetate		
	–	H	N	–	H	N	–	H	N	–	H	N
4662	1	1	0.7	0.07	0.1	0.07	0.1	0.1	0.07	0.02	0.02	0.07
4699	–	1	0.25	–	0.1	0.07	–	0.1	0.07	–	0.02	0.02

B.

	15 mM Acetate			50 mM Acetate			100 mM Acetate			200 mM Acetate		
	–	H	N	–	H	N	–	H	N	–	H	N
4662	1	1	0.7	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
4699	–	1	0.25	–	0.25	0.25	–	0.25	0.25	–	0.25	0.1

–: SA plates; H: SA plates + hemin; N: SA plates + nisin.

Values: colony size relative to PJ4662 on 15mM acetate.

tions the energy recycling by acetate efflux is no longer possible, due to the lack of an acetate gradient as the driving force [7], and these data then indicates that acetate efflux is not the explanation for the complementation by hemin. Inhibition in the form of decreased colony size was observed for both strains when acetate levels where raised far above 15 mM as expected from the anti microbial activity of acetic acid.

Can malolactic fermentation replace the H^+-ATPase activity in *L. lactis*?

In the so-called malolactic fermentation [5,6], cells can generate energy due to proton export by a malate/lactate antiporter coupled with oxidative phosphorylation. To test whether malolactic fermentation could reconstitute the growth of the H^+-ATPase-negative strain we supplemented SA agar plates with 5 mM malate. No growth of the mutant strain PJ4699 however was detectable when hemin or nisin was absent. This result indicates that malolactic fermentation alone is insufficient to allow for generation of a proton gradient in *L. lactis*.

Are there more respiratory processes hidden in *L. lactis*?

The earlier data from *E. faecalis* [2] has indicated that an actual process of oxidative phosphorylation can be reconstituted by supplying cofactors to the growth medium: normally 4 moles of ATP can be obtained from one mole of glucose solely via substrate level phosphorylation, but in this organism, hemin increased the ATP yield to 5 mol ATP/mole glucose. Mannitol fermentation in the presence of hemin increased the ATP yield even further to 6 mol ATP/mol mannitol; it appears that the extra electron pair of mannitol compared to glucose can be used in oxidative phosphorylation.

Our data support that this may also be so for *L. lactis* and we therefore tested if the strains used in this study should have a higher ATP yield on mannitol compared to glucose. Unfortunately both strains grew rather poorly on mannitol, possibly because this substrate is exotic to this organism, and we can therefore not make any conclusions from this experiment.

The supplementation of SA agar plates with another co-factor of the electron transport chain, ubiquinone (Q_{10}), did not in itself result in any detectable growth of strain PJ4699. Also the addition of ubiquinone and hemin together in liquid SA medium did not reveal an obvious increase in biomass compared to SA medium with just hemin. However, these findings could be due to the very low water solubility of this compound, and we are presently testing ubiquinones with lower molecular weight.

Hemin addition eliminates nisin inhibition in the wild type strain

The nisin induced expression system is widely used for activating gene expression in lactic acid bacteria. However, often there is a strong side effect of nisin in the sense that it inhibits the growth of the bacteria. Interestingly, in our experiments, hemin appeared to abolish the inhibiting effect of nisin in liquid culture. When the wild type *L. lactis* strain, PJ4662 was grown with nisin, and with or without hemin, we observed a beneficial action of hemin on biomass yield and slightly on growth rate. PJ4662 grown in the presence of nisin had a 5 to 15% lower final biomass compared to cultures without nisin. Adding hemin to the media resulted in identical biomass yields and growth rates of cultures, independent of the presence or absence of nisin. Since nisin is known to act as an uncoupler of the membrane potential in bacteria, this result further supports the hypothesis that hemin activates a process involved in proton pumping.

Concluding remarks

We report here that hemin enables the growth of a *L. lactis* mutant deficient in H^+-ATPase. This result supports the hypothesis that hemin, to some extent, reconstitutes an alternative route for proton pumping in this organism. An interesting question is now whether hemin also allows for the lactococci to perform oxidative phosphorylation, i.e., whether the H^+-ATPase starts to act as an ATP synthase, turning lactococci into lactocoli.

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