JOURNAL OF BACTERIOLOGY, Nov. 2001, p. 6707–6709 Vol. 183, No. 22
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.22.6707–6709.2001

Hemin Reconstitutes Proton Extrusion in an H^+-ATPase-Negative Mutant of *Lactococcus lactis*

LARS M. BLANK,[1] BRIAN J. KOEBMANN,[2] OLE MICHELSEN,[2] LARS K. NIELSEN,[1] AND PETER R. JENSEN[2]*

Department of Chemical Engineering, University of Queensland, 4072 Brisbane, Australia,[1] and Section of Molecular Microbiology, BioCentrum, Technical University of Denmark, DK-2800 Lyngby, Denmark[2]

Received 30 May 2001/Accepted 20 August 2001

H^+-ATPase is considered essential for growth of *Lactococcus lactis*. However, media containing hemin restored the aerobic growth of an H^+-ATPase-negative mutant, suggesting that hemin complements proton extrusion. We show that inverted membrane vesicles prepared from hemin-grown *L. lactis* cells are capable of coupling NADH oxidation to proton translocation.

The (F_1F_0) H^+-ATPase complex plays an important role in the free energy transduction of living cells. In organisms such as *Escherichia coli* and *Bacillus subtilis,* which contain respiratory chains, the primary role of the enzyme is to synthesize ATP. This process is driven by the proton gradient resulting from respiration when these organisms are supplied with an electron acceptor (3). In organisms that lack a respiratory chain or in the absence of electron acceptors, the enzyme generates a transmembrane proton gradient, and this process is driven by ATP hydrolysis. The anaerobic bacterium *Lactococcus lactis* also possess an F_1F_0-ATPase complex. The enzyme is here involved in the extrusion of protons, driven by ATP hydrolysis, in order to generate the necessary driving force for solute transport and to maintain an acceptable intracellular pH (8, 17).

The *atp* operon encoding the membrane-bound H^+-ATPase of *Lactococcus lactis* subsp. *cremoris* MG1363 (4) was recently cloned and characterized (9), and a mutant strain was constructed, designated PJ4700, in which the native *atp* promoter was replaced by the nisin-inducible *nisA* promoter (NICE system [1]). This strain is H^+-ATPase negative under noninduced conditions of the *nisA* promoter and cannot grow on chemically defined medium (6) (Fig. 1A), which shows that the H^+-ATPase is essential for growth of *L. lactis* under standard cultivation conditions (9).

Hemin restores the growth of an H^+-ATPase-negative mutant of *L. lactis*. Chemically defined SA medium (pH 7), supplemented with 10 g of glucose and an additional 15 g of agar per liter for plates, was used for all experiments (6). Plates were incubated at 30°C overnight. The wild-type strain used for comparison was PJ4662 (MG1363 with pAK80 [5]), in order to exclude any effect of the erythromycin used to select strain PJ4700. Under these circumstances PJ4700 was completely dependent on nisin for growth (9).

Interestingly, the addition of hemin (5 μg/ml in plates; Sigma H-2250) to the medium completely restored the aerobic growth of strain PJ4700 in the absence of nisin (Fig. 1B). In contrast, hemin did not restore growth when plates were incubated anaerobically (data not shown).

These results strongly suggest that hemin complements a respiration-dependent proton transport system other than the H^+-ATPase. Early reports on the effect of hemin addition to a variety of lactic acid bacteria like *Enterococcus faecalis* and *Lactobacillus mesenteroides* are reviewed in London (11). Briefly, reconstituted cytochromes were found in cells grown in the presence of hemin, and at least in one strain (*E. faecalis* subsp. *zymogenes*), the reconstituted cytochromes resulted in an increased ATP yield on glucose of 20% (13). More evidence for the functionality of the reconstituted cytochromes was presented by Ritchey et al. (14), who showed NADH-driven ATP generation as an indication of proton transport in vesicles from *E. faecalis* subsp. *zymogenes* grown in hemin-containing media.

Sijpesteijn (16) reported cytochrome reconstitution and NADH oxidase activities in *L. lactis* (previously *Streptococcus lactis*); no indication was given as to whether this activity was linked to proton extrusion. In a later study, 134 streptococcal strains were screened for cytochrome-like NADH oxidase activity, and it was found that three of nine *L. lactis* strains and one of two *L. lactis* subsp. *diacetylactis* but none of the *L. lactis* subsp. *cremoris* (four strains tested) had cytochrome-like NADH oxidase activity (15).

We measured the reduced minus oxidized absorbance spectrum of our *Lactococcus* strain by the opal glass transmission method. The spectrum confirmed that the gamma (Soret) band appears at 425 to 427 nm, the beta band at 553 to 555 nm, and the alpha band at 574 to 576 nm, showing that this strain is capable of forming cytochromes when hemin is provided in the growth medium (data not shown).

***L. lactis* requires proton extrusion for growth.** One might argue that bacteria growing in well-buffered medium could, in principle, survive and grow without a proton gradient, which could then explain the growth of the H^+-ATPase-negative mutant in the presence of hemin. Experiments with *Enterococcus hirae* suggested that the Na^+-ATPase was responsible for an ATP-dependent generation of a membrane potential (7).

To test whether a proton gradient was actually restored in the mutant cells growing in the presence of hemin, we added the uncoupling agent 2,4-dinitrophenol (DNP) to SA plus hemin plates. At a DNP concentration of 10 mM but not 5 mM

* Corresponding author. Mailing address: Section of Molecular Microbiology, BioCentrum, Technical University of Denmark, Building 301, DK-2800 Lyngby, Denmark. Phone: 45 45–252510. Fax: 45 45–932809. E-mail: Peter.R.Jensen@BioCentrum.DTU.DK.

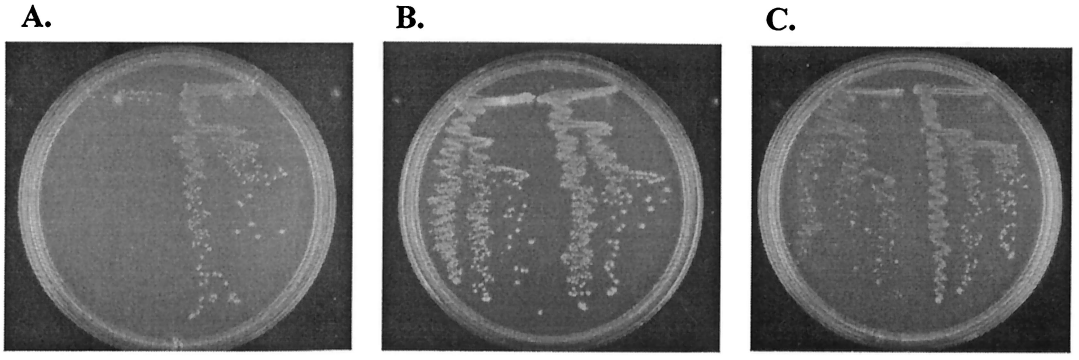

FIG. 1. Hemin effect on growth of *L. lactis* strains PJ4700 (left) and PJ4662 (right). (A) SA agar plate + erythromycin (5 μg/ml). (B) SA agar plate + hemin (5 μg/ml) + erythromycin (5 μg/ml). (C) SA agar plate + nisin (16 ng/ml) + erythromycin (5 μg/ml).

(Fig. 2A), growth of strain PJ4700 was abolished (Fig. 2B), which suggested that a reconstituted proton gradient via hemin was eliminated by the use of this uncoupler. Interestingly, strain PJ4662 still grew in the presence of 10 mM DNP even though growth was significantly reduced. The most reasonable interpretation of this result is that the H^+-ATPase is sufficiently active to overcome the influx of protons carried by 10 mM DNP. The respiration-driven efflux can overcome the influx of protons carried by 5 mM DNP but not by 10 mM DNP.

End product efflux cannot account for generation of proton gradient in H^+-ATPase-negative mutant. The group of Konings reported that end product efflux could generate a proton gradient by a symport mechanism (for a review, see reference 10). The stoichiometry in moles of protons per mole of end product of this transport is highly dependent on the end product gradient and can vary from 0.9 (a net import of protons) to 2.0. Under standard laboratory conditions, i.e., in the absence of hemin, lactate efflux apparently cannot generate a proton gradient sufficient for growth, since the growth of PJ4700 requires nisin under these conditions (9). However, the addition of hemin might reconstitute a cytochrome-like NADH-oxidase activity (a redox sink) that would allow a switch in pyruvate metabolism. Such a switch from lactate to acetate production due to NADH oxidase overexpression was reported previously (12). The efflux of alternative end products could then result in the generation of a proton gradient.

To test the hypothesis of end product efflux's generating a proton gradient, we grew the H^+-ATPase-negative mutant, PJ4700, on a series of plates containing hemin and increasing concentrations (15 to 200 mM) of potential end products (acetate, lactate, and acetoin). The growth of strain PJ4700 was not abolished under the conditions tested, even when the end product concentration reached 200 mM (data not shown). Under these conditions, energy recycling by end product efflux is no longer possible, due to the lack of a gradient as the driving force (18). These data show that end product efflux cannot account for the maintenance of a proton gradient in the H^+-ATPase-negative mutant.

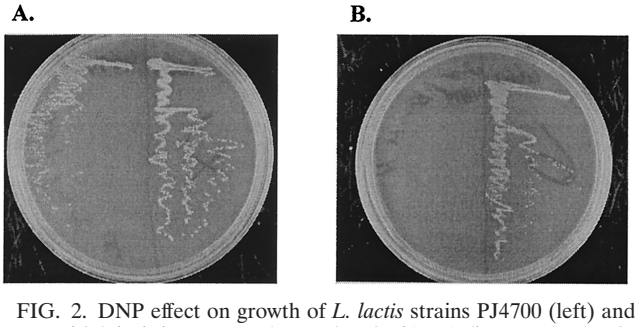

FIG. 2. DNP effect on growth of *L. lactis* strains PJ4700 (left) and PJ4662 (right). (A) SA agar plate + hemin (5 μg/ml) + erythromycin (5 μg/ml) + 5 mM DNP. (B) SA agar plate + hemin (5 μg/ml) + erythromycin (5 μg/ml) + 10 mM DNP.

NADH-driven proton translocation in inverted membrane vesicles of hemin-grown cells. The data presented thus far provide very strong indications that hemin reconstitutes respiratory processes and that these processes are capable of generating a proton gradient in *L. lactis*. We decided to see if it was possible to measure NADH-driven proton extrusion activity directly in inverted membrane vesicles.

L. lactis cells were grown as aerobic batch cultures in M17 medium supplemented with 1% (wt/vol) glucose and 2 μg/ml erythromycin and in the absence or presence of hemin. At an optical density at 600 nm (OD_{600}) equal to 0.6, 100 ml of culture was harvested by centrifugation and resuspended in assay buffer to an OD_{600} of 10. Vesicles were prepared essentially as described by Friedl et al. (2), except that 20 mM MOPS-KOH, 10 mM $MgCl_2$, and 300 mM KCl (pH 7.3) was used as the assay buffer. Briefly, cell suspensions were incubated overnight in the presence of 0.1 mg/ml lysozyme. The following day, cell suspensions were processed with a French press (40,000 lb/in^2) in the presence of 0.2 mM phenylmethylsulfonyl fluoride (protease inhibitor).

The vesicles were incubated in the presence of the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA), which binds to energized membranes and is quenched by a pH gradient. Finally, the rate of fluorescence quenching was measured using a spectrofluorophotometer with excitation at 410 nm and emission at 490 nm after addition of 0.25 mM NADH. The vesicles prepared from hemin-grown cells indeed gave rise to fluorescence quenching, which indicated that the interior of the vesicles was being acidified (Table 1). In contrast, the vesicles from cells grown without hemin showed virtually no indications of proton-pumping activity. Experiments with wild-type cells (PJ4662) and the H^+-ATPase-negative mutant gave similar results.

TABLE 1. Measurements of proton extrusion activity of inverted membrane vesicles from *L. lactis* cells grown with and without hemin

Strain	Hemin (5 μg/ml) added	Rate of fluorescence quenching[a]
PJ4662	Yes	5.0 to 7.7
PJ4662	No	−0.8 to 0.1
PJ4700	Yes	5.0 to 6.8

[a] Range of values obtained from two to three determinations (relative units per minute).

In conclusion, the data presented in this paper show that hemin enables the growth of an *L. lactis* H^+-ATPase-negative mutant by reconstituting an alternative proton transport system in this organism. We also demonstrated that vesicles prepared from hemin-grown cells are capable of coupling NADH oxidation to proton translocation; therefore, a good candidate for this function is a cytochrome using molecular oxygen as the terminal electron acceptor.

We thank Regina Schürmann for excellent technical assistance.

This work was supported by C. Hansen A/S, the Danish Academy of Technical Sciences (ATV), and the Australian Research Council. L.B. was funded by a UQ Travel Award.

REFERENCES

1. **deRuyter, P., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62:**3662–3667.
2. **Friedl, P., J. Hoppe, R. P. Gunsalus, O. Michelsen, and H. U. Schairer.** 1983. Membrane integration and function of the three F0 subunits of the ATP synthase of *Escherichia coli* K-12. EMBO J. **2:**99–103.
3. **Futai, M., and H. Kanazawa.** 1983. Structure and function of proton-translocating adenosine triphosphatase (F0F1): biochemical and molecular biological approaches. Microbiol. Rev. **47:**285–312.
4. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
5. **Israelsen, H., S. M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen.** 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector, pAK80. Appl. Environ. Microbiol. **61:**2540–2547.
6. **Jensen, P. R., and K. Hammer.** 1993. Minimal requirements for exponential growth of *Lactococcus lactis.* Appl. Environ. Microbiol. **59:**4363–4366.
7. **Kakinuma, Y., and K. Igarashi.** 1995. Electrogenic Na^+ transport by *Enterococcus hirae* Na(+)-ATPase. FEBS Lett. **359:**255–258.
8. **Kobayashi, H.** 1985. A proton-translocating ATPase regulates pH of the bacterial cytoplasm. J. Biol. Chem. **260:**72–76.
9. **Koebmann, B. J., D. Nilsson, O. P. Kuipers, and P. R. Jensen.** 2000. The membrane-bound H^+-ATPase complex is essential for growth of *Lactococcus lactis*. J. Bacteriol. **182:**4738–4743.
10. **Lolkema, J. S., B. Poolman, and W. N. Konings.** 1995. Role of scalar protons in metabolic energy generation in lactic acid bacteria. J. Bioenerg. Biomembr. **27:**467–473.
11. **London, J.** 1976. The ecology and taxonomic status of the lactobacilli. Annu. Rev. Microbiol. **30:**279–301.
12. **Lopez F. de Felipe, M. Kleerebezem, W. M. de Vos, and J. Hugenholtz.** 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. J. Bacteriol. **180:** 3804–3808.
13. **Pritchard, G. G., and J. W. Wimpenny.** 1978. Cytochrome formation, oxygen-induced proton extrusion and respiratory activity in *Streptococcus faecalis* var. *zymogenes* grown in the presence of haematin. J. Gen. Microbiol. **104:**15–22.
14. **Ritchey, T. W., and H. W. Seeley.** 1974. Cytochromes in *Streptococcus faecalis* var. zymogenes grown in a haematin-containing medium. J. Gen. Microbiol. **85:**220–228.
15. **Ritchey, T. W., and H. W. Seely, Jr.** 1976. Distribution of cytochrome-like respiration in streptococci. J. Gen. Microbiol. **93:**195–203.
16. **Sijpesteijn, A. K.** 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek **36:**335–348.
17. **Suzuki, T., and H. Kobayashi.** 1989. Regulation of the cytoplasmic pH by a proton-translocating ATPase in *Streptococcus faecalis* (*faecium*): a computer simulation. Eur J. Biochem. **180:**467–471.
18. **ten Brink, B., and W. N. Konings.** 1982. Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. **152:**682–686.