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Bioelectrochemistry 57 (2002) 113–118

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

# Effect of reducing agents on the acidification capacity and the proton motive force of *Lactococcus lactis* ssp. *cremoris* resting cells

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Received 12 April 2001; received in revised form 17 December 2001; accepted 11 January 2002

**Abstract**

Reducing agents are potential inhibitors of the microbial growth. We have shown recently that dithiothreitol (DTT), $NaBH_4$ and $H_2$ can modify the proton motive force of resting cells of *Escherichia coli* by increasing the membrane protons permeability [Eur. J. Biochem. 262 (1999) 595]. In the present work, the effect of reducing agents on the resting cells of *Lactococcus lactis* ssp. *cremoris*, a species widely employed in dairy processes was investigated. DTT did not affect the acidification nor the ΔpH, in contrast to the effect previously reported on *E. coli*. The ΔΨ was slightly increased (30 mV) at low pH (pH 4) in the presence of 31 mM DTT or 2.6 mM $NaBH_4$. In the case of $Na_2S_2O_4$, small amounts (0.9 mM) drastically decreased the acidification range and this product was shown to abolish the ΔpH. These results are discussed in terms of the diversity of action of the chemical reagents and strain sensitivity. 

*Keywords:* Dithiothreitol; Sodium borohydride; Sodium dithionite; Proton motive force; Acidification; Lactic acid bacteria

## 1. Introduction

Optimal redox ranges have been observed for the growth of several microorganisms [1–3]. This has stimulated researches on the use of redox reagents to inhibit the microbial growth [4]. Such compounds are also used in the microbial mediated biotechnological processes such as the reduction of fumarate [5,6], technetium [7] or uranium [8] using lactate, hydrogen or even the electrically reduced neutral red as the electron donor. In these processes, redox compounds make the reaction possible but in the mean time and due to their toxic effect, they alter the cellular metabolism. A better understanding of the mechanisms of action of the reducing reagents on the bacteria is thus of particular interest both to increase the yields of production in the biotechnological processes using microorganisms in extreme reducing environments and to elaborate strategies to inhibit the growth of the undesired microorganisms.

The mechanisms of growth inhibition can be divided into three groups: (i) reduction of medium molecules lowering the accessibility to the substrate or increasing the toxicity of some compounds [9], (ii) modification on the cell surface, altering transports [10] and (iii) decrease in the intracellular oxido-reduction potential modifying, for instance, disulfide bond formations and thus enzyme activity [11] or lowering the cofactor reoxidation. The latter two groups have obvious implication on the cell energetics and particularly on the proton motive force. Recently, we have shown that due to an increased proton membrane permeability, *Escherichia coli* was not able to maintain its intracellular pH in reducing environments [12]. Moreover, such conditions, maybe through the lowered intracellular pH, have an effect on the activity of some enzymes, and following, on the carbon and electron flow [13].

Among the bacteria of great significance in biotechnology, lactic acid bacteria play a major role especially in the food industry. *Lactococcus lactis* spp. *cremoris*, for instance, is used in many dairy processes mainly for its great acidification capacity and for its role in the generation of the aminoacid-derived aroma compounds [14]. It is also used for its ability to produce exopolysaccharides [15] or bacteriocines [16]. Moreover, the generation and physiological significance of the proton motive force of this strain have been widely studied [17–19]. From our previous results [12,13] and from those described in other studies using the reducing compounds [5–8], we can expect a strong impact in the applications of *L. lactis* ssp. *cremoris* when modifying the redox environment. As a first step, we investigated in this study the

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1567-5394/02/$ - see front matter 
PII: S1567-5394(02)00051-8

effect of some reducing agents on the acidification capacity and on the intracellular pH of these lactic acid bacteria encountered in both biotechnological processes and in food contamination.

Since most of the reducing agents are chemo selective, i.e. they are selectively attacking one functional group in the presence of other groups, we used three different compounds, dithiothreitol (DTT), sodium borohydride ($NaBH_4$), and sodium dithionite ($Na_2S_2O_4$). DTT is a compound used to reduce the disulfide bonds or to protect thiols from the oxygen oxidation. $NaBH_4$ is widely used to reduce ketones and aldehydes, and $Na_2S_2O_4$ reduces ketones, aldehydes, and quinones but also double bonds in unsaturated conjugated ketones and diunsaturated acids. It is used notably to reduce the redox enzymes.

We have observed that in contrast to *E. coli*, the intracellular pH of *L. lactis* ssp. *cremoris* was unaffected by DTT and $NaBH_4$. However, these compounds slightly increased $\Delta\Psi$ in the acid conditions. $Na_2S_2O_4$ for its part, even in far lower concentration, affected the ΔpH and inhibited the growth and acidification. We discussed these results in terms of their chemical and biological diversity.

## 2. Experimental

### 2.1. Strain and media

*L. lactis* ssp. *cremoris* SC09 (INRA, Jouy-en-Josas, France) was used in this study. It was precultured on a modified MRS medium [20] containing 10 g/l of lactose as the carbohydrate source. Cells were grown in 150-ml flasks containing 100 ml of medium inoculated with 0.045 g/l ($OD_{580}=0.1$) of cells. For the resting cell experiments, cells were harvested in late log phase, centrifuged for 10 min at 4000 × *g* and washed with potassium phosphate buffer (pH 7.0, 50 mM). For the monitoring of acidification, 110 mg/l ($OD_{580}=0.25$) of cells were suspended in distilled water containing 9 g/l of sodium chloride and 1 g/l of lactose. The acidification kinetics of the resting cells, in the presence of lactose as the only carbon source, was followed at 26 °C with a combined electrode (Inlab 427, Metler-Toledo) connected to a pH-meter (C834-Consort, Bioblock Scientific).

### 2.2. Reducing agents

Dithiothreitol, sodium borohydride, and sodium dithionite were used as the reducing agents at the concentrations used in the previous study with *E. coli* (3 or 13 mM DTT and 2.6 mM $Na_2BH_4$) [12] or when no effect was detected at higher concentrations of DTT (31 mM). $Na_2S_2O_4$ was used at a lower concentration (0.9 mM) able to significantly decrease the oxido-reduction potential. Solutions were prepared before the experiment to avoid the oxidation or degradation of the reactants and the reduced state of the compounds was checked by measuring the oxido-reduction potential with a redox-combined electrode (Pt 4805-DXK, Metler-Toledo) combined to a redox controller (P507 Consort, Bioblock Scientific). Media were deaerated by sparging $N_2$ to avoid the interactions between oxygen and the reducing compounds or electrodes. The response of the electrodes was checked after the use of each reducing compound. Before each measurement, the redox-combined electrode was polished with a 15-μm average diameter aluminum oxide powder to restore the platinum surface. $NaBH_4$ was manipulated under the hood and dissolved in a NaOH (30 g/l) aqueous solution as it decomposes with water more quickly in the acid solution forming the toxic diborane gas and flammable, explosive hydrogen gas. However, it was not used in the acidification experiments because of its ability to react with water forming NaOH.

### 2.3. Proton motive force evaluation

For the intracellular pH evaluation, the probe 5(6)CFDA, SE (5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes, Eugene, OR, USA) was used. This electrically neutral compound is permeant to cell membranes, and upon hydrolysis by the intracellular nonspecific esterases, forms carboxyfluorescein, which has a pH-dependent spectral response. Carboxyfluorescein is preferred to fluorescein because of its extra negative charges enabling a better retention of the probe in cells. Bacteria were charged with the probe (2 μM) in HEPES buffer (50 mM, pH 8) for 30 min. After washing, they were incubated with 1 mM lactose in potassium phosphate buffer (50 mM, pH 7.0) for 30 min to facilitate the efflux of probe excess and, eventually, probe-containing cells were suspended at 340 mg/l ($OD_{580}=0.75$) in TRIS-MES buffer (50 mM) at various pH in the presence or in the absence of the reducing compounds. The dual measurement of fluorescence (Ex: 440 nm (pH independent) and 490 nm (pH dependent), Em: 520 nm) was used to evaluate the intracellular pH, as described in Ref. [21]. Calibration was carried out in the buffer at a different

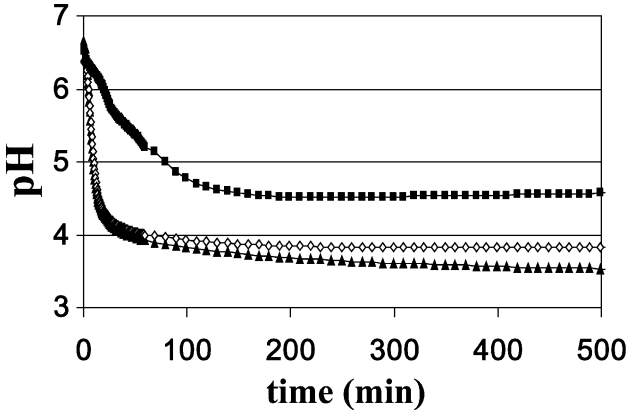


Fig. 1. Extra cellular pH monitoring for resting cells of *L. lactis* ssp. *cremoris* in the presence of dithiothreitol (◇), dithionite (■) or without the reducing agent (▲). Data presented are averages from two or three independent experiments. Standard deviation is less than 10%.

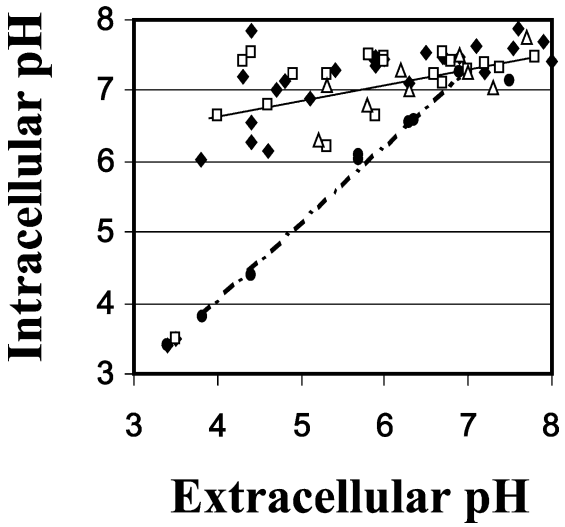


Fig. 2. Intra cellular pH vs. extra cellular pH for resting cells of *L. lactis* ssp. *cremoris* in the presence of dithiothreitol (□), $NaBH_4$ (△), dithionite (●) or without the reducing agent (♦). Data are from at least 10 independent experiments and each point results from one experiment.

pH, with or without the reducing agents, with cells equilibrated with the external medium by incubation with 1 μM nigericin and valinomycin.

The membrane potential ($\Delta\Psi$) was evaluated by monitoring the distribution of the lipophilic cation tetraphenylphosphonium ($TPP^+$) across the cell membrane. This was done by measuring the external concentration with a $TPP^+$ selective electrode. The nonspecific binding of the probe, evaluated using 2% toluene treated cells, was taken into account [22]. Cell concentration in the measured vessel was of $OD_{580} = 2.5$. For calculation, the internal volume of *L. lactis* ssp. *cremoris* cells was supposed to be 2 μl/mg of dry cells, assuming that no significant change occurred in the different conditions. The $TPP^+$ selective electrode and a calomel reference electrode (Radiometer Analytical, Villeurbanne, France) were connected to an ion meter (P507 Consort, Bioblock Scientific) and a chart data recording system. The permeability of the selective membrane to the $TPP^+$ and the response of the electrode were routinely checked particularly in the presence of the reducing agents and the electrode membrane was changed as soon as the response ratio was altered.

The proton motive force ($\Delta p$) was calculated as follows

$$\Delta p = \Delta\Psi - Z\Delta\mathrm{pH}$$

where $Z$ is equal to $2.3RT/F$ (with $R$: gas constant, $T$: temperature (K), and $F = 96\,500$ C mol$^{-1}$).

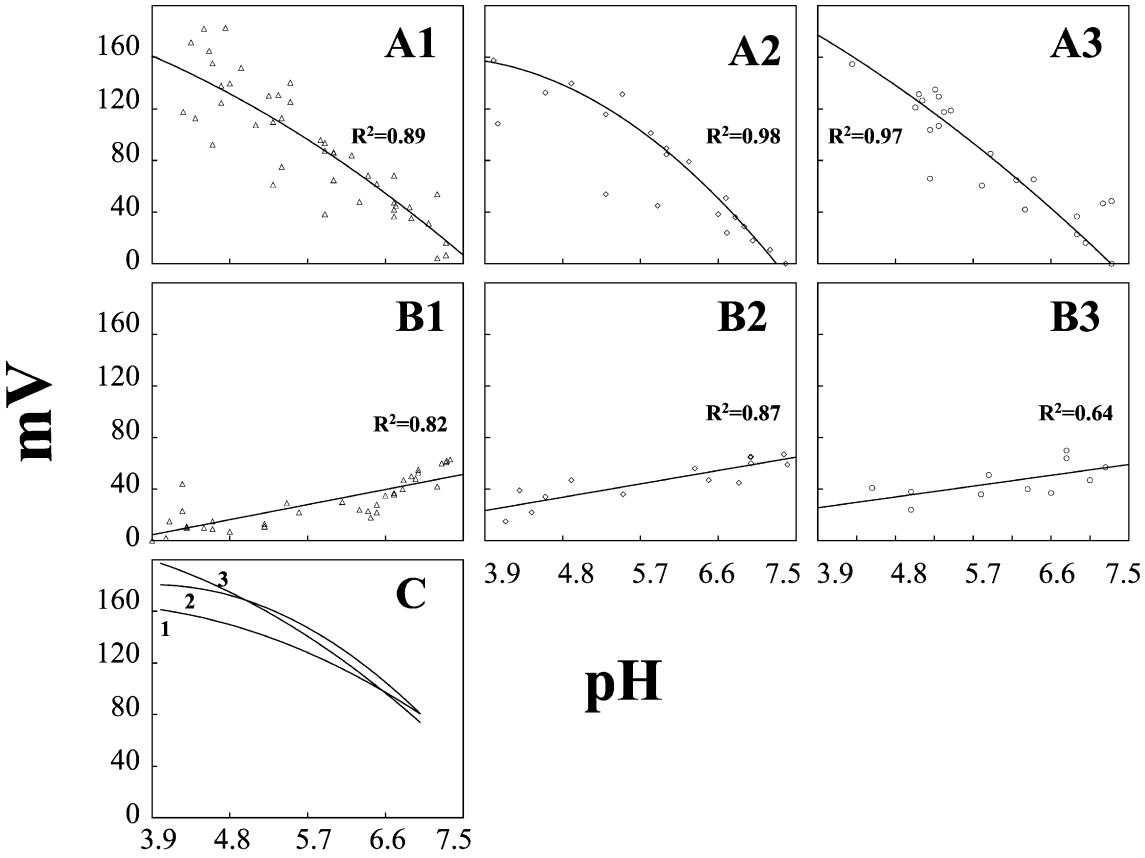


Fig. 3. (A) $Z\Delta$pH, (B) $\Delta\Psi$ and (C) $\Delta p$ for the resting cells of *L. lactis* ssp. *cremoris* with (2) DTT, (3) $NaBH_4$ or (1) without the reducing agent. Data are from at least 10 and 6 independent experiments for $Z$pH and $\Delta\Psi$, respectively. Curve fitting data (second order and first order polynomial for $Z$pH and $\Delta\Psi$, respectively) have been chosen without the physiological significance to calculate (C) $\Delta p$ and $R^2$ are given in the figure.

## 3. Results

As *L. lactis* ssp. *cremoris* is widely used for its acidification capacities, we first investigated the effect of the reducing agents on this property (Fig. 1). We did not use $NaBH_4$ because of its ability to modify the pH by producing NaOH.

In half an hour, pH fell from 6.5 to 4.2 with nearly the same rate without the reducing agent and with DTT. After 30 min, the rates became different and the acidification went on to 3.5 without DTT and to 3.8 with DTT. With dithionite, the pH decreased slowly to 4.5.

To investigate the effect of the reducing agents on the proton motive force, the two components of this force (ΔpH and $\Delta\Psi$) were monitored in a 3.9–7.5 extra cellular pH range and in the presence of DTT (up to 31 mM), $NaBH_4$ (2.6 mM), or $Na_2S_2O_4$ (0.9 mM). Fig. 2 shows the intracellular pH of the resting cells with or without the reducing compounds. Without the redox agents, the intracellular pH was well maintained above pH 6.5, for extra cellular pH was low as 4. For lower values, the intracellular pH decreased to the value of the medium. This pH maintenance was unaffected by DTT or $NaBH_4$, whereas the ΔpH was equal to 0 with $Na_2S_2O_4$. $Z\Delta$pH, the component of the proton motive force varied thus from about 30 mV at pH 7 (low ΔpH) to 160–170 mV at pH 4 (higher ΔpH) with almost no difference in the presence DTT or $NaBH_4$ (Fig. 3A). $Na_2S_2O_4$ had such an effect on the main component of the proton motive force that it did not seem necessary to go further with the $\Delta\Psi$ evaluation, the more so because this product altered the $TPP^+$ selective membrane permeability. Fig. 3B shows the $\Delta\Psi$ of *L. lactis* ssp. *cremoris* resting cells. Without the reducing compounds, the $\Delta\Psi$ value was about 40 mV at near neutral pH and decreased to 0 at pH 4. In the presence of DTT or $NaBH_4$, $\Delta\Psi$ was similar at near neutral pH but was slightly better maintained at lower pH (30 mV at pH 4). The resulting proton motive force ($\Delta p = \Delta\Psi - Z\Delta$pH) is given in absolute value in Fig. 3C. At near neutral pH and without the reducing agents, $\Delta p$ was equal to 90 mV and $\Delta\Psi$ and $Z\Delta$pH both accounted approximately for half of the value. In contrast, at lower pH, $\Delta p$ was enhanced to a value of 170 mV due to the increase in ΔpH and the account of $\Delta\Psi$ was null. $\Delta p$ was even higher at low pH with DTT or $NaBH_4$ as $\Delta\Psi$ was better maintained.

## 4. Discussion

Acidification by *L. lactis* ssp. *cremoris* is a reaction widely used in the dairy processes for the milk gelification which corresponds to the casein precipitation at pH 4.65. The pH decrease is linked to the production of lactic acid from sugar. Growing cells in the MRS medium can acidify to around the pH 4 in excess of lactose (results not shown). The slowing down of the acidification can be due to an alteration of the metabolism by the pH. Lactic acid is a weak organic acid that is not charged at low pH and can easily pass the cell membrane in the protonated form dissociating then at the cytoplasmic pH [19] making the intracellular pH regulation very difficult at low pH. With this lactic acid-derived effect, limits of the acidification and thresholds of the intracellular pH maintenance should be very close. That is what we observed since the acidification stopped between pH 3.5 and 4 and the intracellular pH was maintained for the extra cellular pH above 4. Acidification and the intracellular pH maintenance are linked to the cell energetics. It has been shown, for instance, that in *L. lactis* ssp. *cremoris* (syn. *Streptococcus cremoris*), $\Delta p$ is generated by the lactate efflux [18,23–25] and is thus involved in the ATP synthesis [17]. Moreover, $Z\Delta$pH is, at low pH, the main component of the proton motive force (Fig. 3). This has an implication on the sugar entry inside the cell. Different sugar transport systems have been described in *L. lactis*, a secondary transport with a symport proton/sugar and a PEP–PTS system. For lactose, both transports coexist, a lactose-PTS, being highly inducible [26] and a lactose permease system [27]. Galactose systems can also transport lactose with various affinities [28] and the transporter is coupled to the proton motive force [29]. Major differences exist between the lactose-PTS and galactose-PTS as the first is inhibited by *p*-chloromercuribenzoate and the latter is insensitive to sulfhydric agents [29]. Such diversity might explain the differences encountered in acidification by differences in the sugar transport. Without reducing agents, lactose-PTS would be the main transporter but because of its sensitivity to redox compounds, galactose-PTS would take its place with DTT but not with dithionite which annihilates the proton motive force. In this latter case, a permease with different kinetic properties could eventually transport lactose for near neutral pH.

It can be noted that, contrary to *E. coli*, only a slight effect of DTT and $NaBH_4$ is observable on the intracellular pH of this lactic acid bacteria and these compounds also have a slight stimulating effect on the $\Delta\Psi$.

The sensitivity of *E. coli* to DTT, $NaBH_4$, or dihydrogen has been explained as being due to an increased proton permeability at lower pH [12]. This permeability due to the redox environment could be linked to the modification of the thiol–disulfide balance in the bacterial surface as also discussed by Bagramyan et al. [10]. According to Robillard and Konings [30], such a thiol–disulfide modification has an implication on transport and cell energetics. Although this point is still unclear, more and more results suggest that *E. coli* responds directly to the redox potential [31]. It has been noted for instance that many important functions in the bacteria are catalyzed by redox-sensitive transporters and enzymes whose redox state of disulfide groups could determine their affinity to the substrate [32].

In the case of *L. lactis* ssp. *cremoris*, the effect of reducing agents is weaker. We can expect the same thiol–disulfide balance involvement to be responsible for this effect. The difference in intensity with *E. coli* could be explained by the different surface (Gram-positive vs. Gram-

negative) and the more acidophilic character of the lactic acid bacteria. We can also mention that, recently, Alakomi et al. [33] have shown that lactic acid permeabilizes the Gram-negative bacteria by disrupting the outer membrane which is not present in the lactic acid bacteria.

However, *L. lactis* ssp. *cremoris* is very sensitive to dithionite. This reagent has a chemo selectivity quite similar to $NaBH_4$ but in contrast to borohydride in our study, it had a very strong effect against the ΔpH. It can be noted that, on the contrary to what have long been thought, most cellular membranes are relatively permeable to it [34]. So, although the function conferring its toxicity to $Na_2S_2O_4$ is not clear, it might be linked to its capacity to pass the membrane and react inside the cell.

This work shows that the reducing compounds can have very different effects on the microbial cells depending on the properties of the reagent (chemo selectivity but also the behavior towards membranes) and of the properties of the cell itself (especially membrane, cell wall and transporter system). This diversity of effects results in a diversity of possibilities to use microorganisms in reducing environments. For instance, it might be possible to force a substrate to follow a specific metabolic pathway in order to change the rate or product of the reaction. Thanks to their different effects on the different strains, maybe due to the diversity in transporters systems, reducing compounds could also be used as selection pressure agents.

## Acknowledgements

The authors are thankful to C. Bernard-Rojas for her technical help.

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