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Increasing the Heme-Dependent Respiratory Efficiency of *Lactococcus lactis* by Inhibition of Lactate Dehydrogenase

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The discovery of heme-induced respiration in *Lactococcus lactis* has radically improved the industrial processes used for the biomass production of this species. Here, we show that inhibition of the lactate dehydrogenase activity of *L. lactis* during growth under respiration-permissive conditions can stimulate aerobic respiration, thereby increasing not only growth efficiency but also the robustness of this organism.

Since the first observation of the growth behavior induced by heme in multiple lactic acid bacterial species (1–3), several studies have been carried out to describe this metabolic process in the dairy species *Lactococcus lactis* (4–9). In the presence of heme and oxygen, initial lactococcal growth occurs via fermentation, and when the external pH reduces to approximately 5.3, the transition to respiration occurs (4). It was hypothesized that the physiological reprogramming that occurs during respiration might be a consequence of the shift in the NAD^+/NADH ratio, which allosterically redirects metabolism in favor of enzymes that use either NAD or pyruvate as a substrate (10). The respiration induced by heme ultimately determines the increase in biomass production, due to the improvement of growth efficiency and an extraordinary increase in long-term survival and resistance to oxidative stress (5). These remarkable phenotypes are of industrial relevance, as made apparent by patent applications that are focused on improving the production of starter cultures (11). By analyzing the energy metabolism of *L. lactis* (Fig. 1), it can be hypothesized that the inhibition of fermentative metabolism (normally homolactic fermentation) under respiration-permissive conditions (heme plus oxygen) promotes oxidation of NADH by the electron transport chain via cytochrome *bd* oxidase. In this study, the inhibition of fermentation was achieved by inhibiting the activity of lactate dehydrogenase by using sodium oxamate, an analogue of pyruvate. We used the laboratory strain *L. lactis* subsp. *lactis* IL1403. Unlike other *L. lactis* strains, IL1403 does not demonstrate an observable shift from homolactic to mixed-acid fermentation, which is governed by the sugar consumption rate (12, 13). Nevertheless, the genes of the mixed-acid pathway are present and expressed at higher levels when the IL1403 strain is cultured in the presence of high levels of galactose (13, 14). We hypothesized that under aerobic conditions, in the presence of heme, the inhibition of lactate dehydrogenase by the glycolytic inhibitor sodium oxamate (15, 16) causes a shift in IL1403 metabolism in favor of NAD^+ regeneration by the respiratory chain enzyme NADH oxidase and to a lesser extent by mixed-acid fermentation. An excess of pyruvate should then be directed to acetoin/diacetyl production (Fig. 1) or mixed-acid fermentation, although the latter is less favored under aerobic conditions (12, 17).

Sodium oxamate improves the aerobic growth of *L. lactis* IL1403. *L. lactis* IL1403 was cultivated at 30°C for 10 h in M17 broth (Difco) containing glucose (20 g/liter) without and with heme (5 μg/ml; Sigma-Aldrich) and different concentrations (5, 10, or 20 mM) of sodium oxamate (Sigma-Aldrich). Aerated cultures were maintained in Erlenmeyer flasks with baffles filled to less than a 1/10-volume capacity in a shaking incubator (250 rpm). The spectrophotometric measurement of cell density during the bacterial growth revealed that sodium oxamate resulted in (i) an increase in the yield of the respiratory culture, from an optical density at 600 nm (OD_{600}) of 8.2 to a maximum of 11.0 (Fig. 2), and (ii) a lag-phase delay of close to 1 h when used at the highest concentration. The final pH of the cultures during aerobic nonrespirative growth was 4.50 ± 0.04 (mean ± standard errors of the means), while pH values of 4.93 ± 0.03 and 5.10 ± 0.05 were measured under respirative growth in the presence of heme alone and growth with heme and sodium oxamate (20 mM), respectively. This was expected, i.e., the pH value of the culture in the presence of heme was higher than the pH of the fermenting culture, due to the lower level of lactic acid production, even if in other *L. lactis* strains larger pH differences have been reported (4). These observations led us to hypothesize that strain IL403 is less prone to carry out heme-dependent respiration than other strains of this species. Moreover, the highest culture pH and the lowest lactic acid concentration were measured in the samples where the medium was supplemented with sodium oxamate (Table 1), in accordance with the inhibitory activity of this molecule toward lactate dehydrogenase, as reported for other microorganisms (15, 16). Furthermore, the inhibitory effect of sodium oxamate toward lactate dehydrogenase was tested on crude cell extract of *L. lactis* IL1403 cells collected during the exponential growth phase at an OD_{600} of 2.0, as described previously (18). The enzymatic assay was performed at 30°C for 30 and 60 s in the absence and in the presence of different concentrations of sodium oxamate. After 30 s of incubation, lactate dehydrogenase activity showed a 9%, 95%, and 100% reduction when sodium oxamate was added at final concentrations of 10, 40, and 80 mM, respectively (data not shown). However, after 60 s (data not shown) of incubation, sodium oxamate was also ineffective in inhibiting lactate dehydro-

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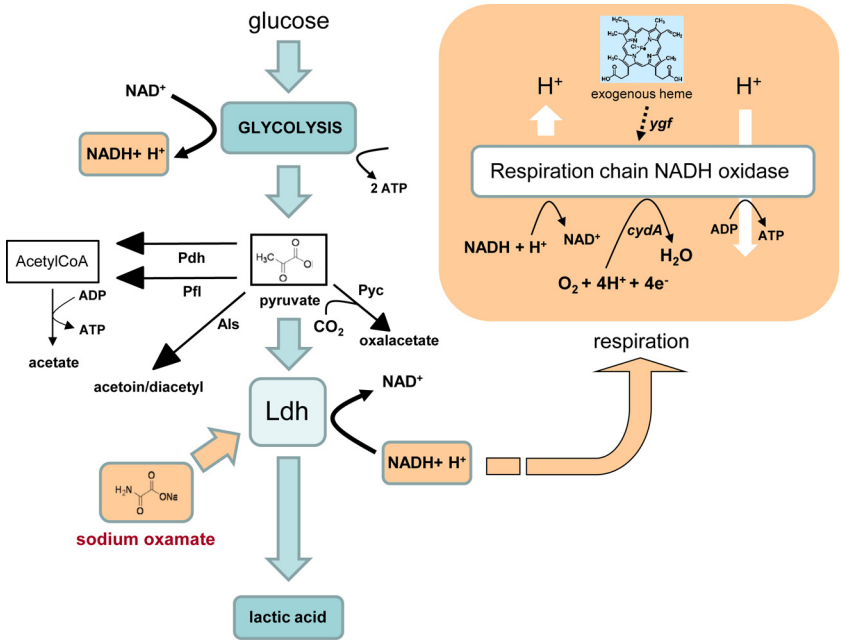

FIG 1 Simplified representation of glycolysis, homolactic, and mixed-acid fermentations and heme-dependent respiration in *Lactococcus lactis* IL1403. Arrows show the metabolic fluxes. Glucose is catabolized to pyruvate via glycolysis, with the production of ATP and NADH. The yellow arrow shows the regeneration of NAD^+ that occurs during heme-dependent respiration. Sodium oxamate competes with pyruvate, lowering the production of lactic acid by lactate dehydrogenase and thus directing the NADH oxidation toward the respiration chain enzyme NADH oxidase. The excess pyruvate can be metabolized by pyruvate formate lyase (Pfl), pyruvate dehydrogenase (Pdh), and acetolactate synthase (Als). The anaplerotic reaction catalized by pyruvate carboxilase (Pyc) is also indicated. *ygf* is the operon involved in heme homeostasis, and *cydA* is the gene for the cytochrome *bd* oxidase subunit I.

genase at the highest concentration tested. It was therefore speculated that sodium oxamate exerts a reversible inhibition on lactate dehydrogenase for *L. lactis*.

The high-performance liquid chromatography (HPLC) analysis of the *L. lactis* IL1403 culture grown aerobically in the presence of sodium oxamate revealed that the glycolytic inhibitor was not metabolized. At the end of *L. lactis* growth, 98.5% of the total amount of oxamate was detected in the spent medium, and the remaining 1.5% was detected in the cell extract, which was obtained from the biomass after mechanical disruption, as previously described (18). As expected, when *L. lactis* IL1403 was cultivated under anaerobic conditions, neither heme nor sodium

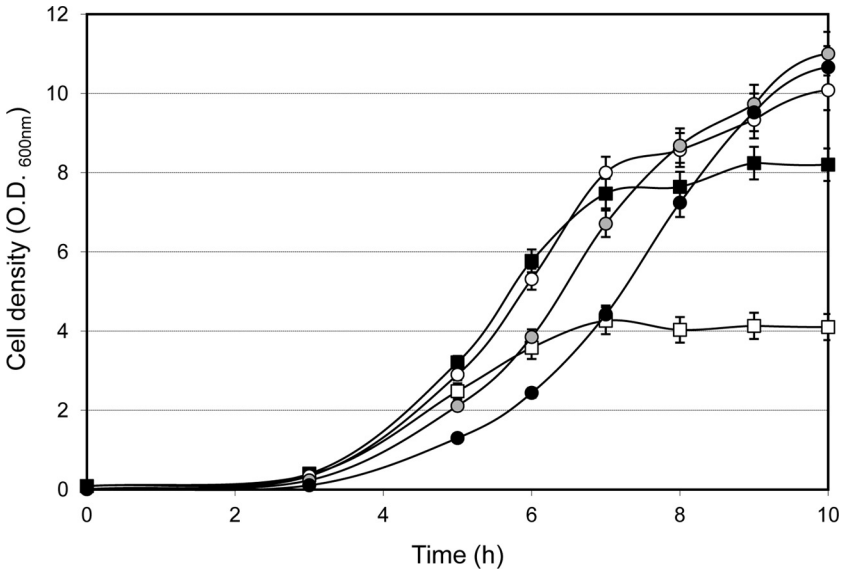

FIG 2 Aerobic growth of *Lactococcus lactis* IL1403 in M17-glucose (2%, wt/vol; white squares), in M17-glucose supplemented with heme (5 μg/ml; black squares), heme and sodium oxamate (5 mM; white circles), heme and sodium oxamate (10 mM) (black circles), or heme and sodium oxamate (20 mM; gray circles). Data points indicate average geometric means of three replicates ± 1 standard deviation, calculated from three independent biological assays for each time point.

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TABLE 1 Metabolic product determinations

Metabolite	Concn (mM) of metabolite under growth condition[a]		
	Ferm	Resp	Resp-Oxa
Lactate	65.0 ± 1.4	51.0 ± 1.5	45.0 ± 1.1
Acetate	7.0 ± 0.2	7.1 ± 0.3	9.4 ± 0.1
Acetoin	5.5 ± 0.1	36.2 ± 0.2	10.2 ± 0.1

[a] Cultures of IL1403 were grown under aerobic conditions (Ferm), respiratory metabolism induced by heme (Resp), and respiratory metabolism induced by heme in the presence of 20 mM sodium oxamate (Resp-Oxa). Values are means ± standard errors of the means.

oxamate showed a positive effect on the biomass amount produced. Anaerobic conditions were obtained by using Erlenmeyer flasks placed in anaerobic jars with a CO_2-enriched atmosphere (18%, vol/vol) obtained using Anaerocult A (Merck KGaA, Milan, Italy).

Effect of sodium oxamate on specific ATP production rate. Respiration is characterized by a high yield of ATP production (the number of units of ATP generated per unit of sugar molecule consumed), and it is associated with a low growth rate but with high biomass production (19). Fermentation, however, is characterized by a high rate of glucose consumption and low biomass production (19). It follows that for *L. lactis*, the ATP production rates during fermentation and heme-dependent respiratory metabolism should then be different. Likewise, the effect of the homolactic fermentation inhibition on the ATP production rate of respiring *L. lactis* cells should be consistent with the incremental change in biomass observed under the same experimental conditions (Fig. 2). The level of intracellular ATP concentration was determined via light emission by the bioluminescent strain 1403-945 (18), which is a derivative of IL1403. In that strain, in the presence of D-luciferin, light emission by firefly luciferase is dependent on the intracellular ATP production rate (18). To measure the light emission, *L. lactis* 1403-945 cultures growing under three growth conditions (aerobic fermentation, heme-dependent respiration, and heme-dependent respiration in the presence of sodium oxamate) were sampled in triplicate after every 30 or 60 min of incubation and dispensed as 100-μl aliquots into 96-well white microtiter plates. We then immediately added the substrate D-luciferin at a final concentration of 0.2 mM (Sigma-Aldrich) to the wells and measured the luminescence signal with a Victor3 luminometer (PerkinElmer, Monza, Italy). The rate of ATP production per unit of time (dATP/d*t*) under the three growth conditions tested showed a similar overall trend (Fig. 3). The differences observed were mainly due to the maximum dATP/d*t* value reached under each growth condition, with the highest value measured in the culture to which heme and sodium oxamate were added to the medium (Fig. 3). Interestingly, the maximum dATP/d*t* value was measured in the early exponential phase of growth during fermentation (OD_{600}, 0.4) and in the late exponential phase of growth (OD_{600}, 2.3 to 2.7) during respiratory metabolism (Fig. 3). The maximum dATP/d*t* value, shown in Fig. 3A, was therefore related to the highest fermentation efficiency (f_{max}), while the maximum dATP/d*t* value shown in Fig. 3B and C was related to the highest respiratory efficiency (r_{max}). It was thus speculated that sodium oxamate affects cell bioenergetics, pushing the metabolism toward a high respiratory efficiency.

IL1403 aerobic cultures, grown for 24 h, as described previously, were subjected to dry weight determinations, and glucose consumption was determined by HPLC as previously described. The yields of biomass production (Y_b; g of dry weight per mol of glucose consumed) of aerated cultures of *L. lactis* IL1403 were 30.1 ± 0.5 for aerobic nonrespiring culture, 33.8 ± 0.8 for the aerobic heme-supplemented culture, and 41.2 ± 0.7 for the aerobic heme and sodium oxamate supplemented culture, thus confirming the role of sodium oxamate in directing cellular metabolism toward energetic pathways characterized by a high ATP yield, i.e., respiration.

Sodium oxamate extends the heme-induced respiratory metabolism of *L. lactis* IL1403 and increases robustness. *L. lactis* cultures carried out in a 2-liter working volume bioreactor (Prelude; Pierre Guerin Technologies, France) under batch conditions, revealed that sodium oxamate greatly extended oxygen consumption, thus confirming its role in reducing fermentation and increasing the respiratory metabolism (data not shown). This effect caused a 68% increase in cell density (the OD_{600}). Moreover, survival in broth culture during storage at 4°C (robustness) was extended well beyond that of fermenting or heme-respiring cul-

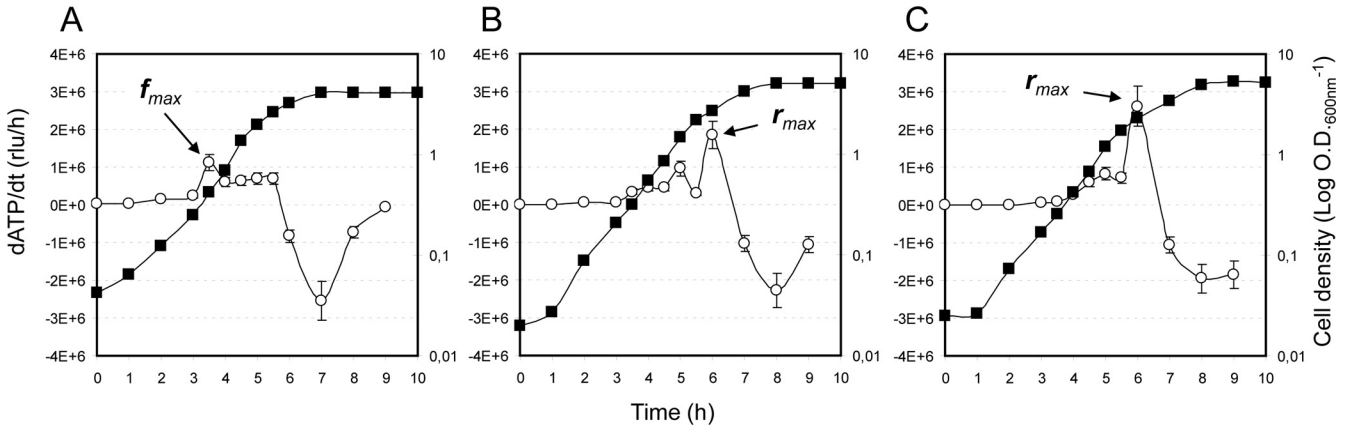

FIG 3 ATP production rate (dATP/d*t*) expressed as light emission during the aerobic growth of *Lactococcus lactis* 1403-945 in M17-glucose (2%, wt/vol) (A), in M17-glucose supplemented with heme (5 μg/ml) (B), or heme and sodium oxamate (20 mM) (C). White symbols represent light emission values. Black symbols represent cell densities. Data points indicate average geometric means of three replicates ± 1 standard deviation, calculated from three independent biological assays for each time point.

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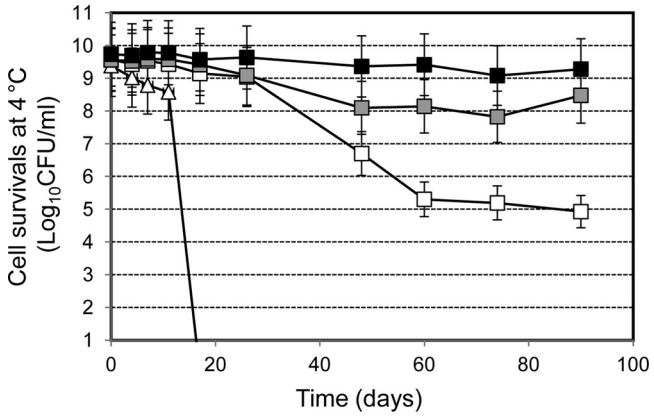

FIG 4 Bacterial cell survival for cells grown for 24 h and then stored at 4°C. Samples were removed for plating at time 0 corresponding to 24 h of growth and thereafter. Cell survival is expressed as CFU/ml in M17-glucose (2%, wt/vol; white triangles), or in M17-glucose supplemented with heme (5 μg/ml; white squares), heme and sodium oxamate (10 mM; gray squares), or heme and sodium oxamate (20 mM; black squares). Data points indicate average geometric means of three replicates ± 1 standard deviation, calculated from three independent biological assays for each time point.

tures (Fig. 4). Surprisingly, aerobic cultures grown in the presence of heme and sodium oxamate survived efficiently, with a less-than-1-log viable population reduction after 90 days at 4°C, whereas the absence of sodium oxamate resulted in an approximate 4-log reduction in the viable population (Fig. 4).

Sodium oxamate redirects central carbon metabolism, as revealed by transcription and metabolite analysis. Total RNA was extracted from cells collected from aerated cultures maintained in Erlenmeyer flasks with baffles when cultures reached an OD_{600} of 1.5, as previously described (18). The evaluation of the relative expression levels of *ygfA* (involved in heme homeostasis) (9), *ldh* (for lactate dehydrogenase) (14), *cydA* (for the cytochrome *bd* oxidase subunit I) (9), *pfl* (for pyruvate-formate lyase) (14), *pycA* (for pyruvate carboxylase), *pdhB* (for the pyruvate dehydrogenase E1 component beta subunit) (14), *als* (for alpha-acetolactate synthase), *amtB* (for the ammonium transporter), and *glnB* (for the nitrogen regulatory protein P-II) was carried out by quantitative reverse transcription-PCR, following a previously described protocol (18). The results were normalized using the *L. lactis tuf* gene, which encodes the elongation factor TU, using as the reference condition the fermenting culture. Under each condition, we performed relative quantification of gene expression in triplicate with the cDNA synthesized from two independent RNA samples. PCRs were carried out as previously described (18) on a CFX96 thermocycler (Bio-Rad Laboratories, Milan, Italy). Data were expressed as the normalized expression ($\Delta\Delta C_T$) ± the standard error of the mean. The results obtained (Table 2) confirmed that *ygf* operon transcription was strongly induced only when the medium was supplemented with heme, while *cydA* and *als* transcription levels were independent of the presence of heme or sodium oxamate. The *pfl* and *pycA* genes, coding for enzymes that utilize pyruvate as substrate, did not show transcription effects based on the culture condition used, thus suggesting that in the presence of sodium oxamate, pyruvate metabolism is redirected to other pathways. According to previous observations (9), despite the fact that *als* was not differentially expressed, acetoin was detected at different concentrations under aerobic fermentative metabolism, heme-dependent respiration, and heme-dependent respiration in the presence of sodium oxamate (Table 1). Considering the extensive changes in acetoin concentrations under the three culture conditions tested and the lack of changes in the expression levels for the *als* gene (Table 2), we hypothesized that part of the pyruvate-consuming fluxes might be controlled at the metabolic level, possibly via the redox balance, as previously suggested (9). During respiratory metabolism induced by heme, we detected a significant increase in *pdhB* transcription, in accordance with previous observations (8, 9), confirming the role of the pyruvate dehydrogenase complex in NADH recycling. Interestingly, the presence of sodium oxamate determined a further increase of *pdhB* transcription, thus suggesting a metabolic shift toward energetically advantageous acetate production (the acetate pathway produces one ATP molecule, which constitutes an energetic gain for the cell compared to lactic acid production) (Fig. 1). Indeed, this increase in acetate production was observed (Table 1). We therefore speculated that the inhibition of lactate dehydrogenase by sodium oxamate results in the redistribution of metabolic flux toward the production of acetate. Moreover, according to Pedersen et al. (9), *amtB* and *glnB* were upregulated during respiratory metabolism (Table 2). These two genes are involved in a channel for ammonium capture that is active under ammonium limitation and feeds into the glutamine synthetase pathway. Surprisingly, the presence of sodium oxamate resulted in reduced expression of *amtB* and *glnB*, even if the levels of their transcripts remained higher than those detected during fermentation (Table 2). The increases of *amtB* and *glnB* transcripts were previously explained via a hypothesis involving a greater demand for nutrients during heme-dependent respiration (9). Consequently, the lower expression levels of *amtB* and *glnB* genes in the presence of sodium oxamate appear to be in disagreement with the increase of biomass obtained using this glycolytic inhibitor and should be further investigated.

Conclusions. This study highlighted the potential usefulness of enzyme-specific inhibitors to direct the energetic metabolism of *L. lactis*. The reversible inhibition of lactate dehydrogenase, obtained by supplying heme-containing media with the glycolytic inhibitor sodium oxamate, was able to increase the efficiency of the heme-dependent respiratory metabolism of *L. lactis* IL1403 by reducing homolactic fermentation. The measurement of substrate

TABLE 2 Transcription analysis results

Gene	Function	Relative expression[a]		
		Ferm	Resp	Resp-Oxa
ygfA	ABC transporter, ATP-binding protein	1.0 ± 0.1	12.0 ± 1.0	10.4 ± 0.7
cydA	Cytochrome *bd* oxidase subunit I	1.0 ± 0.3	0.3 ± 0.6	0.6 ± 0.7
ldh	Main lactate dehydrogenase (20)	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
pfl	Pyruvate-formate lyase	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.1
pdhB	Pyruvate dehydrogenase E1 component	1.0 ± 0.1	2.6 ± 0.2	4.1 ± 0.2
pycA	Pyruvate carboxylase	1.0 ± 0.2	0.9 ± 0.1	1.1 ± 0.1
als	Alpha-acetolactate synthase	1.0 ± 0.1	0.8 ± 0.2	0.6 ± 0.1
amtB	Ammonium transporter	1.0 ± 0.1	14.8 ± 1.0	4.1 ± 1.1
glnB	Nitrogen regulatory protein P-II	1.0 ± 0.1	16.4 ± 2.0	5.0 ± 1.5

[a] Cultures of IL1403 were grown under aerobic conditions (Ferm), respiratory metabolism induced by heme (Resp), or respiratory metabolism induced by heme in the presence of sodium oxamate at 20 mM (Resp-Oxa). Values are means ± standard errors of the means.

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consumption during the growth of *L. lactis* IL1403 revealed that sodium oxamate increased the yield of biomass production during heme-dependent respiratory metabolism, thus improving also the long-term survival compared to that obtained with the use of the heme alone. The metabolic strategy described here for the increase of the respiration capacity of *L. lactis* opens the door for more efficient production of this industrial bacterium and for the development of novel applications in which a large amount of biomass and prolonged survival are beneficial. The inhibitor compound sodium oxamate is clearly not immediately suitable to add to the media of culture productions where the resulting cells are to be used in food fermentations. One alternative to still achieve an improved yield could be, for example, to make a conditional mutation in the *ldh* gene, ideally through non-genetically modified organism methods.

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