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Engineering the central pathways in *Lactococcus lactis*: Functional expression of the phosphofructokinase (*pfk*) and alternative oxidase (*aox1*) genes from *Aspergillus niger* in *Lactococcus lactis* facilitates improved carbon conversion rates under oxidizing conditions

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ABSTRACT

The present work describes a novel central pathway engineering method that has been designed with the aim to increase the carbon conversion rates under oxidizing conditions in *L. lactis* fermentations. The nisin producer *L. lactis* ATCC11454 strain has been genetically engineered by cloning a truncated version of the phosphofructokinase gene (*pfk13*), along with the *pkaC*, encoding for the catalytic subunit of cAMP-dependent protein kinase, and the alternative oxidase (*aox1*) genes of *A. niger*. Functional expression of the above genes resulted in enhanced PFK activity and the introduction of AOX activity and alternative respiration in the presence of a source of heme in the substrate, under fully aerobic growth conditions. The constructed strain is capable of fermenting high concentrations of glucose as was demonstrated in a series of glucostat fed-batch fermentations with glucose levels maintained at 55, 138 and 277 mM. The high maximum specific uptake rate of glucose of 1.8 mM s^{-1} g CDW^{-1} at 277 mM glucose is characteristic of the improved ability of the microorganism to handle elevated glucose concentrations under conditions otherwise causing severe reduction of PFK activity. The increased carbon flow through glycolysis led to increased protein synthesis that was reflected in increased biomass and nisin levels. The *pfk13*–*pka*C–*aox1*-transformant strain's fermentation at 277 mM glucose gave a final biomass concentration of 7.5 g/l and nisin activity of 14,000 IU/ml which is, compared to the parental strain's production levels at its optimal 55 mM glucose, increased by a factor of 2.34 for biomass and 4.37 for nisin.

1. Introduction

The industrial importance of lactic acid bacteria along with their attractive characteristics, such as the small genome size (~2–3 Mb) and simple energy and carbon metabolism, have made them promising targets of metabolic engineering strategies. Such strategies have mainly focused either on rerouting of pyruvate metabolism to produce important fermentation end products [1,2] or on the complex biosynthetic pathways leading to the production of exopolysaccharides and vitamins [3,4]. Attempts to manipulate the central carbon metabolism or respiration in these bacteria are rather limited.

L. lactis shows homolactic metabolism when growing in rapidly metabolized sugars with more than 90% of the metabolized sugar being converted to lactic acid. Deviation from homolactic fermentation is observed under aerobic conditions [5,6] or during the metabolism of galactose or maltose [7,8]. Regulation of glycolysis and the shift between different fermentation modes have attracted increased attention and have been studied extensively [9–13]. Application of metabolic control analysis revealed a number of factors that control the flux through glycolysis [6]. Among them, phosphofructokinase (PFK) was identified as the key regulatory enzyme of the glycolytic flux [6,13,14]. In addition, processes outside the glycolytic pathway itself, like glucose transport and the ATP consuming reactions, were also found to have a significant influence on the control [13].

It was shown in our earlier work [15] that enhancement of PFK activity, through engineering of the primary metabolic pathway in *L. lactis*, increases the glucose transport capacity and the glycolytic flux. *L. lactis* strains harboring a truncated version of *A. niger*'s *pfk* gene (*pfk13*) were able to grow successfully, under the identified as optimal microaerobic conditions (5% DOT), in glucostat cultures with 277 mM glucose-concentration levels at which the parental strain was apparently inhibited.

As mentioned above, the flow of carbon towards different pathways that lead to the formation of various types of products

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depends on the levels of available oxygen in the substrate. The sensitivity of *Lactococcus* spp. in elevated oxygen levels is well known. However, an important and for long time overlooked trait of this organism is its ability to perform respiratory metabolism in the presence of heme and under aerobic conditions [16] although there is no evidence for the presence of an alternative respiration pathway and AOX activity. We have shown in an earlier work [17] that expression of the alternative oxidase gene (*aox1*) of *A. niger* in *L. lactis* confers on this organism cyanide-resistant growth, which in turn is sensitive to salicylhydroxamate (SHAM). The cyanide-insensitive, alternative respiratory pathway operates in the transformed *L. lactis* and improves significantly the microorganism's tolerance to oxidizing stress conditions as it induces increased biomass production, reduced lactate formation, and accumulation of large amounts of the lantibiotic nisin.

It could be expected therefore, that a combined engineering strategy aiming at both enhancement of glycolysis and introduction of alternative respiration would allow the organism to handle more efficiently elevated glucose concentrations under aerobic conditions. Triple-transformant strains were prepared by introducing plasmids to them containing a cassette with the truncated version of *pfk*, *pfk13*, along with the *pkaC* gene, encoding for the catalytic subunit of cAMP-dependent protein kinase, and the *aox1* genes of *A. niger*. The reason for cloning the *pkaC* along with *pfk13* is the need of phosphorylation of the product-enzyme of the truncated gene in order to become activated. The applied cloning strategies and transformation results for *L. lactis* have been reported in our earlier publications [18,19]. Extending our work, we report here the results of aerobic glucostat fed-batch fermentations carried out with transformant strains harboring the set of three *A. niger* genes.

2. Methods

2.1. Microbial strains and culture conditions

The nisin producer *L. lactis* ssp. *lactis* ATCC11454 is the parental strain. The transformants used were the *aox1* containing strain, as was described in [17], and the triple-transformant strain containing a cassette with t-*pfk* (*pfk13*), *pkaC* and *aox1* from *A. niger* A60, as was described in [18]. Preparation of competent cells and electrotransformation was done as described by Papagianni et al. [19].

L. lactis was routinely grown in the chemically defined medium (CDM) used in an earlier work by Papagianni et al. [20], supplemented with the heme source hemin (1.25 μg/ml) (Sigma) and 5 g/l glucose. Flask-cultivation was carried out in 150 ml Erlenmayer flasks (50 ml medium), at 30 °C and 100 rpm.

2.2. Fermentations

Bioreactor-cultivation was carried out in a stirred tank bioreactor – BIOFLO 110, New Brunswick Scientific – with a working volume of 2 l. The agitation system of the reactor consisted of two 6-bladed Rushton-type impellers (52 mm), operated at the stirrer speed of 100 rpm. Process temperature was maintained at 30 °C and culture pH was maintained at 6.8 by automatic addition of 5 M NaOH solution. Fully aerobic conditions were maintained by sparging the reactor with air at the rate of 1 vvm to ensure that DOT was more than 90% of saturation. The bioreactor was inoculated with cells from precultures at the end of the exponential phase, grown in Erlenmeyer flasks as described above, buffered with 15 g/l K_2HPO_4. At inoculation time, initial biomass concentration in the reactor was 1 mg/l.

Fermentations were carried out in the mode of glucostat fed-batch cultures, as has been described earlier [15]. The working volume was 2 l for the batch cultures whereas the initial volume for the glucostat cultures was 1.7 l and the final 2 l. The feed consisted of a glucose solution (150–300 g/l) that was added at a rate of 5–10 $g\,l^{-1}\,h^{-1}$, designed to keep the glucose concentration relatively constant, the variation not exceeding 5%. The concentration of glucose in the feeding solution and the feeding rate were estimated on the basis of the rate of glucose consumption in batch cultures performed with various initial glucose concentrations. The initial volume of medium in fed-batch cultures contained all other chemicals apart from glucose, in amounts estimated for 1.7 l of medium. Experiments were carried out at the following glucose levels: 55, 138 and 277 mM (10, 25 and 50 g/l, respectively). Samples were taken at inoculation time and at 1-h intervals until runs' termination at 20 h. All runs were carried out in triplicate (mean values presented) and repeated if experimental variation exceeded 5%.

2.3. Analytical methods

2.3.1. Analysis of fermentation products and glucose

Biomass, lactic acid, residual glucose, and nisin concentrations were determined as described by Papagianni et al. [20]. Pyruvate and acetate concentrations were determined as described by Papagianni et al. [13].

2.3.2. Enzyme assays

Phosphofructokinase (PFK) was assayed by the method previously described by Le Bloas et al. [21] and modified by Even et al. [9], as it was applied in our earlier work [15].

2.3.3. In vivo respiration assays

In vivo respiration assays were carried out as described in our earlier work [17]. To distinguish cyanide-sensitive respiration, 1 mM KCN was added to the medium and AOX activity was detected by the addition of 2.5 mM SHAM. The effective doses of inhibitors were determined by titrating the reagents against the control according to Kirimura et al. [22]. The respiratory rate of the t-*pfk*–*pka*C–*aox1*-transformant and the control strain was calculated as the oxygen consumption per OD_{600}. Overexpression of the *A. niger aox1* allowed respiration after addition of KCN and this feature was used to select strains with actively expressed *aox1*.

2.3.4. Preparation of cell extracts

Bacteria were harvested by centrifugation (16,000 × *g*, 30 min, 4 °C) at an OD_{600} of 1, washed twice with 0.85% NaCl, and suspended in 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol [23]. The bacteria were disrupted ultrasonically (20 kHz) at 0 °C for 36 cycles of 5 s. Cell debris was removed by centrifugation (13,000 × *g*, 10 min, 4 °C).

2.3.5. Determination of intracellular sugar-phosphates

Extracts were prepared from cultures at an OD_{600} of 1 according to Andersen et al. [11,14]. Glucose-6-phosphate dehydrogenase from yeast was obtained from Boehringer (Mannheim, Germany), while phosphoglucose-isomerase and fructose bisphosphate aldolase were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Intracellular concentrations of sugar-phosphates were calculated assuming that 1 g (dry weight) corresponds to 1.67 ml of intracellular volume and an OD_{600} of 1 corresponds to 0.25 g (dry weight) per liter.

3. Results and discussion

3.1. Functional expression of PFK and AOX

The expression cassette t-*pfk*–*pka*C–*aox1* of 4127 bps (Fig. 1) was cloned into a pTRKH3-based vector and the recombinant plasmid pTR3 (pTR-t–*pfk*–*pka*C–*aox1*) of 9042 bps (Fig. 2) was used to transform *L. lactis* ATCC11454 cells [19]. According to Southern blot analyses, different copy numbers of the set of three genes were inserted in *L. lactis* transformants. The strain selected for the present work carried 20 copies each of *pfk*13, *pkaC* and *aox1* genes. Table 1 shows the results for specific activities of PFK for the t-*pfk*–*pka*C and the t-*pfk*–*pka*C–*aox1*-transformants in glucostat cultures with glucose maintained stable during fermentation at 55, 138 and 277 mM.

It has been shown by Gaudu et al. [24,25] that induction of SHAM-sensitive AOX activity varies with the growth phase of the culture and cells of the exponential growth phase develop high rates of SHAM-sensitive O_2 uptake. Therefore, the rate of oxygen uptake was measured during the late exponential phase of growth and the results are shown in Fig. 3. Transformants showed cyanide-insensitive and SHAM-sensitive respiration. In parental strain cells, the oxygen uptake was completely inhibited by addition of KCN, whereas in transformants expressing *aox1* the addition of SHAM inhibited respiration. Also, it is obvious from the figure that AOX activity was induced by the inhibitor of cytochrome oxidase KCN, a factor that reduces the activity of the mitochondrial electron chain. This cyanide-insensitive respiration, indicated as KCN-resistance of the transformant pTR3, clearly indicates that *A. niger aox1* is encoded and successfully expressed in *L. lactis*.

3.2. Fermentations

In our previous work with cloning and expression of *aox1* of *A. niger* into *L. lactis* ATCC11454, results were reported from glucostat