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ORIGINAL PAPER

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Metabolism associated with raised metabolic flux to sugar nucleotide precursors of exopolysaccharides in *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Abstract Exopolysaccharide (EPS) metabolism was studied in a galactose-negative strain of *Lactobacillus delbrueckii* subsp. *bulgaricus*, using two different approaches. Firstly, using both the parent strain and a chemically induced mutant with higher yield and specific productivity of EPS than the parent, comparative information was obtained relating to enzyme activities and metabolite levels associated with EPS formation when grown on lactose. Under continuous culture conditions ($D = 0.10\ h^{-1}$), the higher metabolic flux towards EPS formation in the mutant strain relative to the parent appeared to be mediated by raised levels of UDP-glucose pyrophosphorylase (UGP). Marginally raised UDP-galactose 4-epimerase (UGE) activity in the mutant strain suggested that this enzyme could also play a role in EPS overproduction. The second approach involved investigating the effect of growth rate on sugar nucleotide metabolism in the parent, as it is known that EPS production is growth-associated in this strain. UGE activity in the parent strain appeared to increase when the growth rate was elevated from 0.05 to 0.10 h^{-1}, and further to 0.35 h^{-1}, conditions that can be associated with higher levels of metabolic flux to EPS formation. Concurrent with these increments, intracellular ATP levels in the cell were raised. In both investigations glucose-6-phosphate accumulated pointing to a constriction at this branch-point, and a limitation in the flow of carbon towards fructose-6-phosphate or glucose-1-phosphate. The changes in metabolism associated with enhanced flux to EPS provide guidance as to how the yield of *Lactobacillus delbrueckii* subsp. *bulgaricus* EPS can be improved.

Keywords Exopolysaccharide · *Lactobacillus delbrueckii* subsp. *bulgaricus* · Metabolism

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Introduction

Exopolysaccharides (EPSs) from lactic acid bacteria (LAB), which are GRAS organisms, represent a group of polymers that have potential for application as food additives or functional food ingredients, and may confer health benefits. Investigations involving mouse models, for example, have suggested that EPSs possess immunostimulatory, antitumoural, and cholesterol-lowering activity [21–23]. EPSs from LAB are presently most commonly used in the dairy industry, and are used to improve the rheology and textural quality of fermented products such as yoghurt. A need therefore exists to improve the productive capacity of LAB for EPS formation, as well as to induce structural changes, which would demonstrably improve the functional characteristics of the polymers. However, the production of these polymers is confined by the productive capacity of the LAB, which are largely anaerobic. Engineering changes to the compositional structure of EPS as well as the economic production of these polymers by LAB for food applications will rely upon an understanding of the associated biosynthetic pathways.

The biosynthesis of EPS production can be broken into four groups of reactions, viz. sugar transport into the cell, synthesis of sugar-1-phosphates, synthesis of sugar nucleotides and conversion into the EPS repeating unit, and EPS polymerization and export from the cell [24] (Fig. 1). The potential for exerting control over enzymes associated with EPS formation exists at any of these levels, and mutants lacking enzymes of any group fail to synthesize EPS [25, 38].

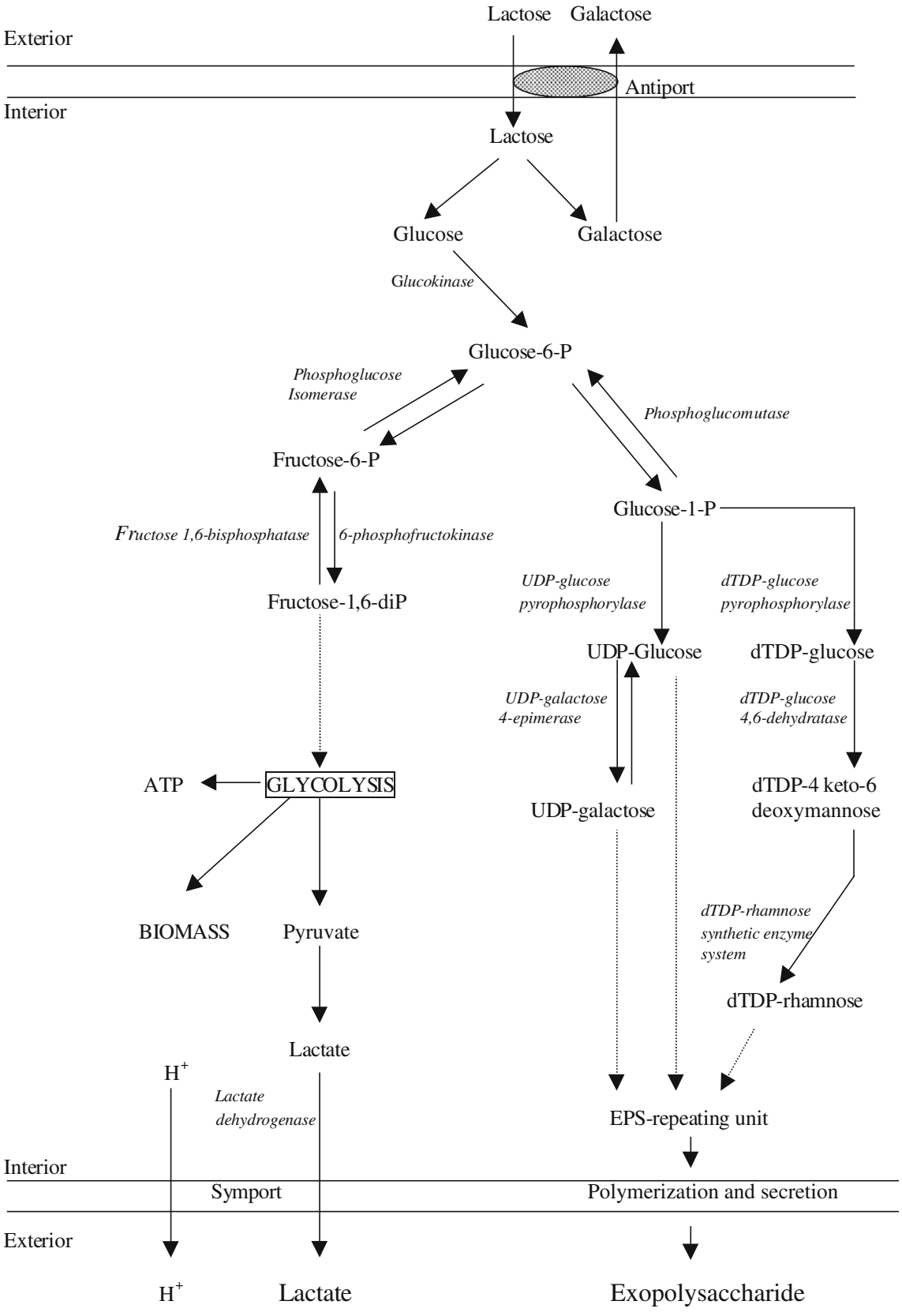

Fig. 1 Simplified scheme of the bioreaction network leading to exopolysaccharide synthesis in *Lactobacillus delbrueckii* subsp. *bulgaricus*

Knowledge of intracellular concentrations of metabolites complements the measurement of enzymatic activities, and is a valuable tool in promoting targeted improvements of metabolites [18]. In the present study, the pools of intracellular metabolites between glucose-6-phosphate and sugar-nucleotide synthesis, as well as associated enzyme activity levels in a strain of *Lb. delbrueckii* subsp. *bulgaricus*, were compared with a chemical-mutant of this strain, known to demonstrate higher yields and rates of formation of EPS and lactate [44]. The glucose-6-phosphate node closely links the catabolic pathways of glucose breakdown, and the anabolic network of EPS production, while the glucose-1-phosphate branch point links the distribution of carbon between the production of sugar nucleotides for the glucose, galactose and rhamnose components of EPS (Fig. 1). By comparing the levels of intracellular metabolites together with related enzyme activities, deductions could be drawn about the contributing role of enzymes to the enhancement of carbon flow from glucose-6-phosphate to the sugar-nucleotide precursors of EPS formation, in a strain with an enhanced specific yield of EPS.

The EPS-producing strain in this investigation, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, is "homolactic", utilizing the glucose moiety of lactose, and excreting the residual galactose from the cell. This pattern of utilization is consistent with most of the dairy strains of *Lb. delbrueckii* subsp. *bulgaricus* [29]. Lactose uptake in strains that are galactose-negative occurs mostly via a lactose/galactose antiport system [6]. The lactose is split via a β-galactosidase into glucose, which is converted to lactate by the Embden–Meyerhoff pathway, and into galactose that is exported from the cell. Although the channelling of some galactose-carbon into the formation of the EPS biosynthetic pathway is possible [43], the wholly galactose-negative phenotype allowed for the discrete study of the biosynthetic pathways via a single port of entry of carbon (viz. via glucose-6-phosphate), leading to the associated sugar-nucleotide precursors of the EPS-repeating unit.

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2483 produces EPS in a growth-associated manner such that EPS formation increases with increasing growth rate [44]. Because of this phenomenon, the effect of different growth rates on the bifurcation of carbon flux at glucose-6-phosphate between the catabolic pathways of glycolysis and the anabolic pathways of sugar-nucleotide and EPS formation could also be studied (Fig. 1).

Materials and methods

Fermentation medium

The medium used was that described by Kimmel and Roberts [20] and modified by the replacement of the glucose component with lactose [43]. Casein hydrolysate (Bacto Casitone) was used as the nitrogen source. For the inoculum, the medium was prepared in separate, double-strength volumes of lactose and the remainder of the nutrients as described previously [43], adjusted to pH 6.0, and pooled after steam-sterilization. Fermentation medium (20 l) was prepared by sterilizing separately 1.5 l of a solution of lactose.H_2O (421.0 g), and 18.5 l of a solution of the remainder of the nutrients combined with antifoam (Bevaloid, Rhodia, 15 ml) in distilled and deionized water in a 20 l carboy (sterilization by steam for 90 min at 121°C). Loss of volume due to venting of the vessel after sterilization was restored by the aseptic addition of the sterile water.

Bacterial strains and inoculum

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. The fermentation inoculum was prepared, as described previously, by inoculating 150 ml of medium with a 1.0 ml aliquot from a working cell bank, preserved at −80°C, and incubating at 37°C for 24 h on an orbital shaker at 100 rpm [44]. The mutant strain (E2483M) was obtained previously by screening of chemically mutagenized strains for acidifying ability, mucoidy, ropiness, and EPS production [45].

Fermentation conditions

Continuous culture experiments (1,400 ml working volume) were performed using a Bioflo I fermenter (New Brunswick Scientific, NJ, USA). A continuous head pressure of nitrogen was maintained for all experiments. The fermenter was operated initially in a batch mode in order for sufficient cell mass to accumulate prior to commencing the continuous studies. Temperature was maintained at 37°C, agitation was set at 200 rpm, and pH was maintained at 6 by titration with 2 M KOH. Fermentations at each dilution rate were run until steady-state conditions had been reached (constant biomass levels); this was usually achieved after four to five fermenter volumes had been replaced.

Sampling

Sample aliquots (15 ml) were withdrawn aseptically at regular intervals for the determination of biomass and sugar conversion to metabolites. For the enzymatic assays, a 300 ml aliquot of the fermenter volume (at steady state) was rapidly decanted via the overflow weir into a pre-chilled centrifuge bottle on ice. Samples for assay of intracellular metabolites in extracts were removed from the fermenter and extracted using a modification of a rapid system previously described by Guedon et al. [16], Fordyce et al. [10] and Thomas et al. [39]. A sterile syringe containing 1.5 ml of perchloric acid, sealed at the exit with a silicone tube and stopcock,

and pre-drawn to a volume of 15.0 ml under vacuum, was attached to the fermenter sampling port. Fermentation broth under steady-state conditions was rapidly withdrawn from the fermenter vessel upon release of the stopcock; the final $HClO_4$ concentration in the syringe was 0.6 M.

Analyses

Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650 nm to washed dry cell weights. Lactose utilization, galactose secretion and lactate formation were measured in duplicate by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and Waters 2487 UV detector). The compounds were detected using an Aminex HPX-87H, 300×7.8 mm column (Biorad, Richmond, CA, USA), according to the method as previously described [36, 43]. Extracellular polysaccharides in fermentation broth were isolated by a twofold precipitation with chilled ethanol [43]. Sugar concentration (EPS) was measured according to the method of Dubois et al. [8], with dextran as the standard.

Assay of intracellular metabolites

Further treatment of the sample was undertaken according to the method described by Guedon et al. [16] and Fordyce et al. [10]. The extracts were centrifuged (10,000*g*) for 10 min at 4°C, and the supernatants stored at −70°C until assayed.

Assays of the intracellular metabolites glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate were undertaken by a modification of the method of Garrigues et al. [11] using fluorimetric determination of NADPH formed in enzyme assays. Emission was measured at 460 nm after excitation at 350 nm using a Perkin Elmer LS 50B Luminescence Spectrometer. The concentration of NADPH formed was related to fluorimetric intensity by creation of a calibration curve ($R^2 = 0.99$) [5]. To 1,150 µl of a mixture of 500 mM triethanolamine buffer (pH 7.6) containing 15 mM $MgSO_4$ and 4 mM EDTA, 40 µl 10 mM NADP, 760 µl H_2O, and 50 µl metabolite extract, was added 20 µl glucose-6-phosphate dehydrogenase (200 U/ml) in order to commence conversion of G6P. After the reaction had proceeded to completion, 20 µl of phosphoglucose isomerase (PGI) (200 U/ml) was added in order to measure the concentration of fructose-6-phosphate. Upon completion of this reaction, 20 µl phosphoglucomutase (α-PGM) (200 U/ml) was added for the measurement of glucose-1-phosphate. All assays were undertaken in triplicate.

Measurements of uridine-5′-diphosphoglucose (UDP-glucose) and uridine-5′-diphosphogalactose (UDP-galactose) were undertaken by an enzymatic method [19] in which the formation of NADH was obtained by absorbance measurement at 339 nm, at 25°C.

The assay of deoxythymidine-5′-diphosphoglucose (dTDP-glucose) was undertaken according to a modification of the method of Bevill [2]. An extract (50 µl) of dTDP-glucose dehydrase prepared from *Escherichia coli* Strain B56, according to the methods of Gilbert et al. [13] and Wang and Gabriel [42], was incubated at 37°C for 1 min in 1,320 µl Tris buffer (50 mM pH 8.6; 2 mM EDTA) prior to the addition of 150 µl metabolite extract. The mixture was incubated at 37°C for 30 min following which 1,480 µl 0.2 M NaOH was added. The assay mixture was subjected to a further incubation period of 15 min at 37°C, following which the absorbance was measured at 318 nm against a blank which was prepared identically, with the exception that the aliquot of 0.2 M NaOH was added before addition of the metabolite extract.

Preparation of cell-free extracts

Crude enzyme extracts were prepared according to a modification of the method of Escalante et al. [9]. Fermentation broth (280 ml) was centrifuged at 35,850*g* for 40 min at 4°C, and the supernatant fraction discarded. The cell pellet was washed with 0.01 M cold phosphate buffer followed by centrifugation at 35,850*g* for 40 min at 4°C. The pellet was resuspended to a total volume of 28 ml (10× concentration) in 0.01 M phosphate buffer and kept on ice. Glass beads (0.13 mm diameter) were added in a ratio of 5.0 g glass to 1.0 g of cell mass. The cell suspension was homogenized in a Braun MSK homogenizer (3×20 s at 400 rpm) with concurrent cooling using a jet of dry CO_2 flowing over the homogenization chamber so as to prevent loss of enzyme activity. The homogenization procedure was pre-optimized in preliminary experiments. The supernatant fraction was further centrifuged at 35,850*g* for 10 min at 4°C prior to assay.

Enzyme assays

The enzyme assays were undertaken at 37°C, and the formation or disappearance of NAD(P)H was measured at 340 nm ($\varepsilon_{340} = 6{,}220\ M^{-1}\ cm^{-1}$), unless stated otherwise. Protein concentration of the enzyme extracts was determined by the method of Lowry et al. [28]. Enzyme activities were expressed as nanomoles of substrate converted to product over 1 min, per mg of protein in the extract. Measurements were reported as means with standard deviations.

Enzyme assays were performed as follows: α-PGM [15, 34]; dTDPglucose pyrophosphorylase (TGP) and UDP-glucose pyrophosphorylase (UGP) [1, 25]; UDP-galactose 4-epimerase (UGE) [7]; phosphoglucose isomerase assay (PGI) [15]; dTDP-glucose 4,6-dehydratase (TGD) [7]; the dTDP-rhamnose synthetic system [30]; NADH oxidase [27].

Table 1 Fermentation titres and performance characteristics of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483, and a mutant strain thereof, grown in continuous culture at a dilution rate of 0.10 h^{-1}

	Parent strain (NCFB 2483)	Mutant strain
Dry cell weight (g l^{-1})	0.36	0.30
Lactose (residual) (g l^{-1})	0.27	0.41
Galactose (g l^{-1})	10.31	9.88
Lactate (g l^{-1})	9.35	8.51
EPS (g l^{-1})	0.10	0.14
EPS vol. productivity (g l^{-1} h^{-1})	0.010	0.014
$Y_{p/x}$ (EPS) (g g^{-1})	0.29	0.48
$Y_{p/s}$ (EPS) (g g^{-1})	0.01	0.01
EPS specific productivity (g g^{-1} h^{-1})	0.03	0.05
Lactate vol. productivity (g l^{-1} h^{-1})	0.94	0.85
$Y_{p/x}$ (Lactate) (g g^{-1})	26.02	28.62
$Y_{p/s}$ (Lactate) (g g^{-1})	0.45	0.44
Lactate specific productivity (g g^{-1} h^{-1})	2.60	2.86
Galactose vol. productivity (g l^{-1} h^{-1})	1.03	0.99
$Y_{p/x}$ (Galactose) (g g^{-1})	28.68	33.28
$Y_{p/s}$ (Galactose) (g g^{-1})	0.50	0.51
Galactose specific productivity (g g^{-1} h^{-1})	2.87	3.33

All values based on the average of two measurements

Assay of nucleotide pools

Adenosine 5′-triphosphate (ATP) in the perchloric acid extracts was assayed using an assay kit (Sigma Diagnostics ATP kit, Catalog No. 366). Adenosine 5′-diphosphate (ADP) was measured by the method of Guedon et al. [17].

Results

Mutant studies: EPS and sugar nucleotide metabolism at constant growth rate

At a dilution rate of 0.10 h^{-1}, the EPS titre of the mutant was substantially raised relative to the parent strain (Table 1). Values determined for $Y_{p/x}$ (EPS) and EPS specific productivity were elevated in comparison to the parent. The mutant exhibited a similar pattern of enhancement relative to the parent in respect of $Y_{p/x}$ and specific productivity of lactate and galactose. No yield improvements relative to lactose consumed ($Y_{p/s}$) were apparent for these metabolites. Larger intracellular pools of glucose-6-phosphate were present in the mutant strain; however, no obvious differences in the pools of fructose-6-phosphate or glucose-1-phosphate were evident (Fig. 2). Intracellular pools of UDP-glucose and UDP-galactose were substantially lower in the mutant strain; no dTDP-glucose was detected (Fig. 2). Levels of ADP and ATP were higher in the mutant strain (3.2 and 3.0 μmol/g cell weight, respectively) than in the parent strain (0.4 and 1.2 μmol/g).

At a dilution rate of 0.10 h^{-1}, UGP activity was substantially higher in the mutant strain than in the parent (Fig. 3). No marked differences in α-PGM and UGE occurred; however, the activity of the latter was marginally raised relative to the parent strain. NADH-oxidase levels were raised relative to the parent (Fig. 3).

Influence of growth rate on EPS and sugar nucleotide metabolism

At higher growth rates (dilution rates) *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 showed enhanced metabolic flux of carbon towards EPS formation, evidenced by elevated EPS titres, specific productivities and yields (Fig. 4); this has previously been demonstrated for this strain [44] and for the RR strain [12]. The specific production rates of the other principal metabolites of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (lactate and galactose) displayed similar general trends in response to the increasing dilution rates, as determined previously [44]. Calculation of the ratios of EPS to lactate produced, revealed no change in the distribution of carbon toward EPS and lactate between the dilution rates of 0.05 and 0.10 h^{-1} (0.008 for both). An increased ratio (0.021) at 0.35 h^{-1}, however, suggested a redistribution of carbon away from lactate production in favour of EPS formation at this dilution rate.

The levels of glucose-6-phosphate increased significantly between dilution rates of 0.10 and 0.35 h^{-1}, whilst its metabolic product pools diminished with increasing dilution rate (Fig. 2). The pool of dTDP-glucose was significantly raised at the higher dilution rates. No significant changes could be observed for the UDP-glucose pools (Fig. 2). UDP-galactose levels were lower at 0.10 h^{-1} than 0.05 h^{-1} but decreased no further at 0.35 h^{-1}.

Enzyme activities associated with increasing growth rates, at the glucose-6-phosphate and glucose-1-phosphate branch points, are shown in Fig. 3. Activities of TGD and the dTDP-rhamnose synthetic enzyme system were detected at levels too low for comparison. UGE emerged as bearing a marked association with increasing growth rate (Fig. 3), and hence raised levels of EPS production rates (Figs. 3, 4).

Additionally, EPS titre (associated with increasing dilution rate) was compared with ATP and ADP levels

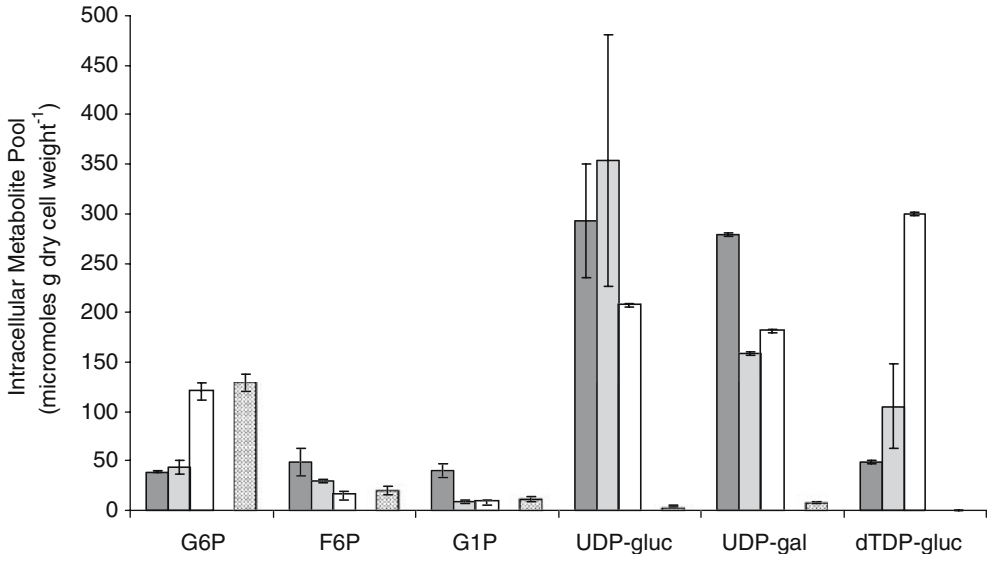

Fig. 2 Intracellular metabolite pools associated with the glucose-6-phosphate and glucose-1-phosphate branch points in *Lactobacillus delbrueckii* subsp. *bulgaricus* strain NCFB 2483, grown at different growth rates in continuous culture (0.05 h^{-1} *dark shaded bar*, 0.10 h^{-1} *light shaded bar*, 0.35 h^{-1} *unshaded bar*), in a semi-defined medium, and a mutant strain thereof (E2483M) under the same conditions at 0.10 h^{-1} (*bar with dots*). Glucose-6-phosphate (*G6P*), fructose-6-phosphate (*F6P*), glucose-1-phosphate (*G1P*), UDP-glucose (*UDP-gluc*), UDP-galactose (*UDP-gal*), dTDP-glucose (*dTDP-gluc*). *Error bars* represent standard deviations

in the cell determined at the respective dilution rates. Generally, energy-rich nucleotides were present at higher levels with increasing EPS formation (Fig. 5).

Discussion

Mutant studies

The mutant strain of *Lb. delbrueckii* subsp. *bulgaricus* (NCFB 2483), although producing a lower biomass than the parent strain, exhibited higher levels of carbon flux to EPS at a dilution rate of 0.10 h^{-1} as shown by the enhanced titre, $Y_{p/x}$ value, and specific productivity of the polymer (Table 1). No substantive difference in the distribution of lactose carbon between the strains was evident, as the corresponding $Y_{p/s}$ values for EPS, lactate, and galactose remained similar. Marginally raised $Y_{p/x}$ and specific productivity levels of lactate and galactose in the mutant reflected an overall increase in flux to these metabolites as well (Table 1).

Raised levels of glucose-6-phosphate in the mutant strain, combined with the fact that the metabolites of this branch-point (fructose-6-phosphate and glucose-1-phosphate) did not accumulate (Fig. 2), point to a metabolic constriction at glucose-6-phosphate. The higher EPS titre and specific yields and productivities of the mutant strain, coupled with the significantly lower levels of the pools of UDP-glucose and UDP-galactose, indicate that a higher level of turnover of these metabolites occurs in comparison to the parent strain (Table 1, Fig. 2). The elevated levels of UGP and UGE activities found in the mutant strain (Fig. 3), relative to the parent, support these observations, and indicate a raised flux of carbon to EPS via UDP-glucose. A correlation between UGP levels and EPS synthesis was also found in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772; however, no association was found with UGE [15]. Neither was an association found between UGE activity and EPS production in ropy or non-ropy Gal^- strains of *Streptococcus thermophilus* [9].

More of the sugar-nucleotides would be available for incorporation into EPS, due to the lower relative biomass levels and less competition for sugar-nucleotides for cell wall synthesis; however, the flux of carbon to EPS cannot be concluded to be controlled by the size of these pools of metabolites alone—more comprehensive flux mapping, supported by experimental data, is needed for the purpose of predicting entire carbon flows to EPS. A similar conclusion regarding the effects of sugar-nucleotide pool-sizes was reached by Ramos et al. [35]. In *Lactococcus lactis* NZ3800, for example, although the levels of UDP-glucose and UDP-galactose were increased fivefold by overexpression of the *pgmU* gene (under nisin-control), no significant changes in EPS production occurred [4].

The lower level of activity of TGP enzyme in the mutant strain coupled with the negligible activity levels of TGD and the dTDP-rhamnose synthetic enzyme system (Fig. 3) implies that the diminished level of dTDP-glucose measured in the mutant arises from the reduced formation of this sugar nucleotide. No enhancement in the activity of α-PGM was evident in the mutant strain (Fig. 3). In contrast, a linear relationship between α-PGM activity and EPS production has been demonstrated in *S. thermophilus* LY03 [7]. The higher PGI levels in the mutant strain (Fig. 3) correlate with the raised glycolytic flux (and flux to lactate).

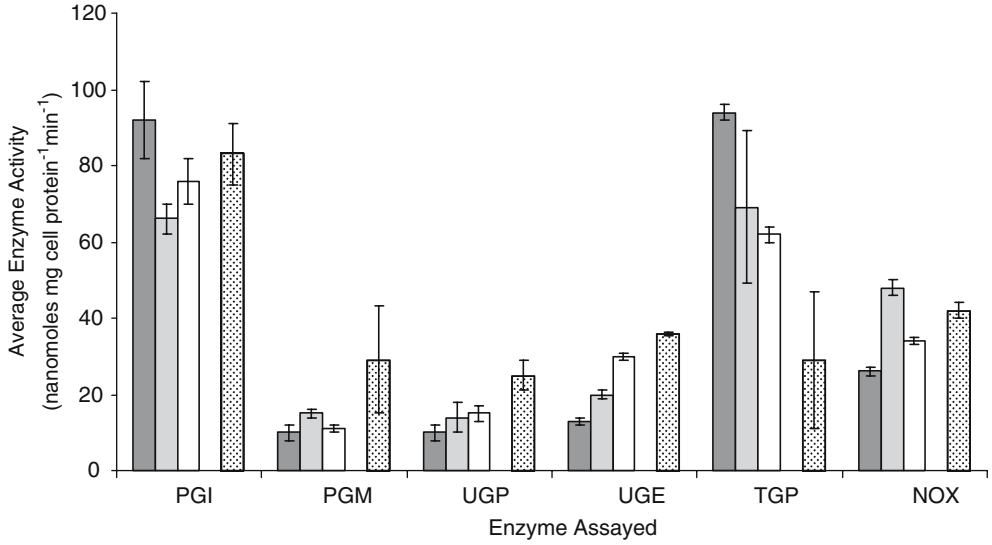

Fig. 3 Activities of enzymes associated with the glucose-6-phosphate and glucose-1-phosphate branch points, and NADH-oxidase in *Lactobacillus delbrueckii* subsp. *bulgaricus* strain NCFB 2483, grown at different dilution rates in continuous culture (0.05 h^{-1} *dark shaded bar*, 0.10 h^{-1} *light shaded bar*, 0.35 h^{-1} *unshaded bar*) in a semi-defined medium, and a mutant strain thereof (E2483M) under the same conditions at 0.10 h^{-1} (*bar with dots*). Phosphoglucose isomerase (*PGI*); α-phosphoglucomutase (*PGM*); UDP-glucose pyrophosphorylase (*UGP*); UDP-galactose 4-epimerase (*UGE*); dTDP-glucose pyrophosphorylase (*TGP*); NADH-oxidase (*NOX*). *Error bars* represent standard deviations. *Note:* dTDP-glucose-4,6-dehydratase (*TGD*) and the dTDP-rhamnose synthetic enzyme system were detected in negligible quantities

The higher levels of ATP and ADP in the mutant strain reflect a higher energy availability for EPS production in comparison to the parent strain.

Growth and EPS-associated metabolic activity

It has previously been demonstrated that the metabolic flux to EPS and lactate is growth-associated in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, increasing up to a growth rate of 0.40 h^{-1} [44]. Because the carbon flux towards EPS formation increases with increasing growth rate in this strain, the metabolic changes in the EPS biosynthetic pathway associated with elevated EPS formation could be measured.

In correspondence with previous results [44], an increment in the dilution rate of the culture of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 from 0.05 to 0.10 h^{-1} resulted in an increase in the flux of lactose-carbon to EPS; a subsequent elevation of the dilution rate to 0.35 h^{-1} raised the flux to EPS further (Fig. 4). This result contrasts with those of Looijesteijn et al. [26], in which the amount of EPS produced by *L. lactis* subsp. *cremoris* per A_{600} unit, grown in chemically defined medium in continuous culture (undefined limiting factor), increased with decreasing dilution rate, a consequence of energy production via glycolysis (production of lactic acid) being completely uncoupled from biomass formation. The elevation of $Y_{p/s}$ values of EPS formation with increasing dilution rate (Fig. 4) demonstrates the diversion of carbon from the formation of other metabolites to EPS formation in response to a changed metabolic status of the cell at higher growth rates. Whether carbon was diverted away from the formation of galactose or lactate is unclear, due to the low levels of EPS produced by the organism; however, the substantial elevation in the ratio of EPS to lactate at 0.35 h^{-1}, relative to the lower dilution rates points to a bias in flux of carbon to EPS relative to lactate. It is likely that the formation of lactate was diminished in favour of EPS production, as the anabolic pathways of EPS formation are linked to the catabolism of glucose (from lactose) by the split of carbon flux at the glucose-6-phosphate branch-point.

The growth-related increase in flux of carbon to EPS and lactate is characterized by the increasing levels of intracellular pools of glucose-6-phosphate, and the diminished pools of its metabolites, fructose-6-phosphate (towards lactate formation) and glucose-1-phosphate (towards EPS formation) (Fig. 2). The metabolic constriction at the glucose-6-phosphate node at the higher dilution rates (growth rates) can be ascribed to the inability of α-PGM and/or phosphoglucoisomerase to efficiently convert the higher intracellular levels of this substrate, even though a small increase in the activity of α-PGM occurred when the growth rate was increased from 0.05 to 0.10 h^{-1} (Fig. 3). Although no significant changes in levels of UDP-glucose were observed at the different dilution rates, the levels of UDP-galactose were reduced between a dilution rate of 0.05 and 0.10 h^{-1} (Fig. 2). Both UDP-galactose and UDP-glucose were determined to be significantly lower in a *L. lactis* EPS-producing strain than in a non-producing one [35]. The fluctuation in intracellular pools of UDP-glucose and UDP-galactose can be ascribed to nodal adjustments to the successive overall increase in flux of carbon to EPS.

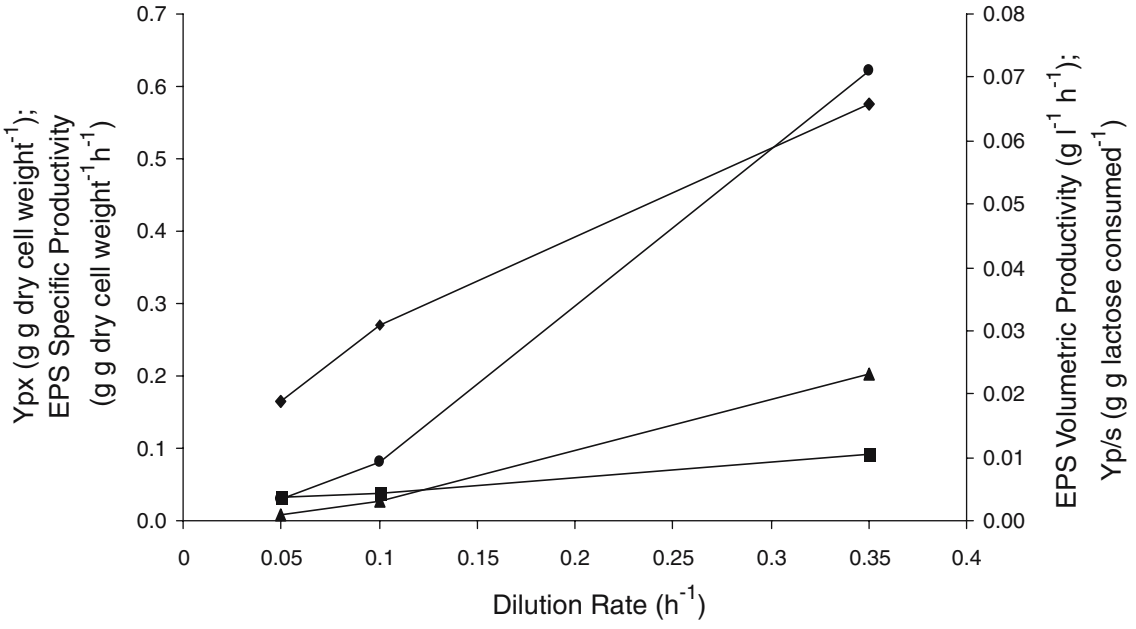

Fig. 4 Exopolysaccharides yields and productivities in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. EPS yield on biomass ($Y_{p/x}$) *diamond*; EPS yield on lactose consumed ($Y_{p/s}$) *square*; EPS volumetric productivity *circle*; EPS specific productivity *triangle*

The significant and progressive increments in UGE activity (Fig. 3) could have occurred as the consequence of an increasing formation of UDP-glucose (directly to the EPS-repeating unit—see Fig. 1), and the requirement of this pathway to maintain a balance between UDP-galactose and UDP-glucose [4]; it is possible that the EPS-assembly process in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 will not recognize a repeating unit which does not contain galactose, and UGE responds in order to maintain this balance. Disruption of the *L. lactis* NIZO B40 UDP-galactose 4-epimerase gene (*galE*) resulted in the abolition of EPS production in *L. lactis* NIZO B40 [3].

The principal enzyme associated with raised levels of EPS was UGE, which demonstrated a trend of progressively increasing activity with increasing growth rate and EPS formation (Fig. 3). In the present study, with increasing growth rate and flux to EPS, similar correlations could not be established for the other enzymes associated with the glucose 6-phosphate and glucose-1-phosphate nodes.

The pools of dTDP-glucose were raised at the higher dilution rates (Fig. 2), showing the inability of the cell to reduce the accumulating levels of this metabolite. This fact, coupled with the negligible activities of the dTDP-glucose 6-hydratase enzyme and the dTDP-rhamnose synthetic enzyme system, indicate that the main avenue of carbon flux to EPS was via the formation of the precursors UDP-glucose and UDP-galactose (see Fig. 1). The composition of EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 would hence be expected to contain lower levels of rhamnose than glucose or galactose; compositional studies undertaken on the EPS produced by the parent strain support this [14].

Enhanced EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, effected by raised growth rates, was accompanied by a raised energy status (Fig. 5). A similar pattern of increasing ATP and ADP levels in conjunction with increasing growth rate has been demonstrated previously in *L. lactis* [37]. It would be worthwhile to pursue further work in order to determine whether ATP levels in the cell can influence the molecular mass of the EPS produced, as has been speculated by Looijesteijn et al. [26]. The mechanism proposed involves an *eps* gene product (*EpsB*) that contains an ATP-binding domain similar to the ExoP-like proteins which have been found to determine EPS chain length in *L. lactis* [41].

The anabolic pathways of EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 are dependent upon glycolysis for the generation of sufficient ATP for the formation of sugar-nucleotides dedicated to cell wall and EPS production. At the higher growth rates, a surplus of ATP would suppress glycolysis, thus limiting the regeneration of NAD^+ produced via pyruvate reduction [40]. A similar finding has been reported for *L. lactis* [33]. It was of interest to see whether an alternative to this mode of NAD^+ regeneration was present viz. by conversion via NADH oxidase (NOX). The presence of NADH oxidase activity has been reported

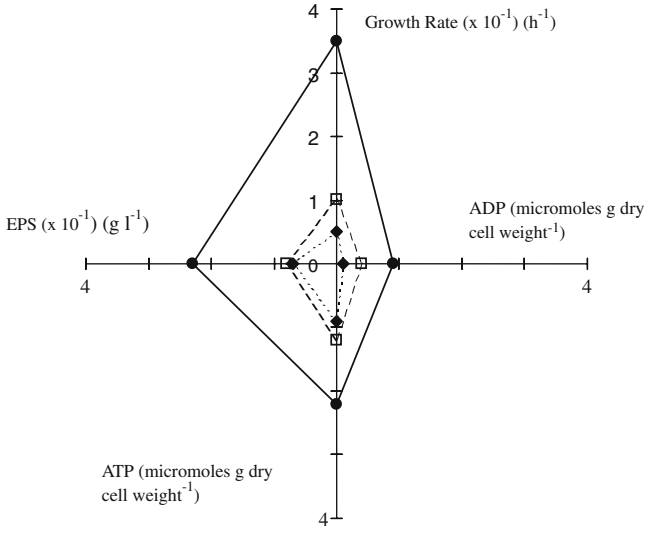

Fig. 5 Relational plot of ATP and ADP levels (micromoles g dry cell weight^{-1}), and formation of EPS (g/l) in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483, grown in a semi-defined medium at steady state in continuous culture at 0.05 h^{-1} (*diamond*), 0.10 h^{-1} (*square*), and 0.35 h^{-1} (*circle*)

previously to occur in aerated and unaerated cultures of *Lb. delbrueckii* subsp. *bulgaricus* B107 [31]. In that investigation, the specific activity of the NADH-oxidase was the same in aerated and unaerated cultures, suggesting that the enzyme was not directly regulated by oxygen. In the present investigation, despite the anaerobic head-pressure applied to the fermentation culture, some dissolved oxygen would have been introduced into the medium with the feed and through constant agitation. The elevated levels of NOX that were associated with raised growth rates and raised levels of EPS formation may be an indication that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 accommodates a higher NAD^+ requirement at higher growth rates by use of this regeneration route. It remains to be demonstrated whether increasing the aeration in the culture would promote the regeneration capacity of the cell for NAD^+ using NADH-oxidase in preference to the reduction of pyruvate. This system has been employed as a strategy to regulate the cofactor balance ($NADH/NAD^+$) in *L. lactis*, thereby diverting carbon flux away from lactate production in favour of other metabolites [27].

Conclusion

In an attempt to clarify the mechanisms of raised carbon flux to EPS in *Lb. delbrueckii* subsp. *bulgaricus* and more specifically, the formation of the sugar nucleotide precursors of the polymer and associated enzyme activities in *Lb. delbrueckii* subsp. *bulgaricus*, an EPS-over-producing mutant was compared with the parent strain (NCFB 2483). A higher metabolic flux towards EPS, in an EPS over-producing mutant of *Lb. delbrueckii* subsp. *bulgaricus*, in comparison to that occurring in its parent strain, appeared to be modulated by raised levels of UGP, and possibly by UGE as well. Significantly elevated levels of glucose 6-phosphate pools in the mutant strain pointed to a metabolic constriction at this branch-point, preventing potentially higher fluxes to glucose-1-phosphate, and sugar-nucleotide precursors.

In the parent strain, a similar constriction at the glucose-6-phosphate branch-point occurred in conjunction with raised growth rates, and associated elevated EPS levels. UGE, UGP and α-PGM, appeared to play a role in the formation of raised EPS production. Elevated levels of ATP were found in conjunction with raised EPS production. This relationship possibly has a bearing on how the glycolytic pathway influences the anabolic pathway of EPS production.

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