Molecular characterization and expression analysis of the dextransucrase DsrD of *Leuconostoc mesenteroides* Lcc4 in homologous and heterologous *Lactococcus lactis* cultures

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The gene encoding the dextransucrase DsrD from the industrial strain *Leuconostoc mesenteroides* Lcc4 was isolated by PCR using degenerate primers recognizing conserved regions present in other dextransucrase-encoding genes from *Leuconostoc* spp. and Southern blot analyses on total genomic DNA. N-terminal sequence analysis of the active protein recovered in the culture showed that the secreted protein of 165 kDa is devoid of a 42 aa prepeptide which is removed post-translationally, most likely by signal peptidase cleavage. Primer extension and Northern blot analysis identified a monocistronic *dsrD* mRNA with two transcription initiation sites. Expression of the dextransucrase DsrD was investigated in pH-controlled fed-batch cultures via Northern blot analysis and enzyme activity measurement during the experiments. Sucrose levels of 20 g l\(^{-1}\) were shown to induce the DsrD biosynthesis around 10-fold. The combination of pH-controlled fed-batch fermentation and Northern analysis clearly showed that *dsrD* expression was related to the growth of the bacteria. *dsrD* was transferred to and expressed in *Lactococcus lactis* MG1363. Controlled fed-batch cultures revealed that active dextransucrase was produced and secreted by the recombinant *L. lactis* strain. The expression was independent of sucrose levels. These results show that dextransucrase can be efficiently expressed and secreted in a non-*Leuconostoc*, heterologous host and is able to drive dextran synthesis.

INTRODUCTION

Glucosyltransferases (EC 2.4) catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The sugar donor can be of diverse nature, e.g. a di- or polysaccharide, a sugar 1-phosphate or a nucleotide diphosphosugar (NDP-sugar). The glucosyltransferases (EC 2.4.1.5) expressed by strains of *Leuconostoc mesenteroides* and oral streptococci are most commonly called dextransucrases and belong to family 70 of glycoside hydrolases (Davies & Henrissat, 1995). They catalyse the transfer of \(\beta\)-glucopyranosyl units from sucrose to acceptor molecules. Thus, two different products can be synthesized: (i) \(\alpha\)-glucans or (ii) oligosaccharides when efficient acceptors (like maltose) are present (Monchois *et al.*, 1999). The dextrans contain more than 50% \(\alpha\text-(1-6)\) linkages and different \(\alpha\)-linked branches, \(\alpha\text-(1-3)\), \(\alpha\text-(1-2)\) or \(\alpha\text-(1-4)\), depending on the glycosyltransferase.

Dextrans have important medical applications in the production of fine chemicals such as plasma substitutes and Sephadex. However, they can also find use for texture improvement in the food industry, e.g. in milk drinks, yoghurts and ice creams. Other ways to improve the texture of fermented products could be to add dextransucrase in an isolated form to the medium during the fermentation process or to use strains of lactic acid bacteria involved in the fermentation process that produce the enzyme and hence the polysaccharides. Despite the industrial importance of dextrans and dextransucrases and the need to produce them in large quantities, little is known about the expression and regulation of dextransucrases in industrially interesting producer strains.

Dextransucrases either are secreted into the culture medium or remain attached to the cell surface of the producing strain. So far, one dextransucrase expressed by *Lc. mesenteroides* NRRL B-512F and two dextransucrases of *Lc. mesenteroides* NRRL B-1299 have been cloned, sequenced and characterized in more detail (Monchois *et al.*, 1996, 1998; Wilke-Douglas *et al.*, 1989). The enzymes are all phylogenetically closely related and are large proteins with an average molecular mass of 160 kDa. They are...
composed of two different functional domains (Monchois et al., 1999). The N-terminal catalytic domain (about 900 aa) is responsible for the cleavage of sucrose (Kato et al., 1992; Mooser et al., 1991; Tsumori et al., 1997). The C-terminal domain (300–400 aa) is composed of a series of homologous directly repeating units and is involved in glucan binding (Giffard & Jacques, 1994). It has been shown that dextranxuscrease expression is constitutive in Streptococcus strains, whereas it is induced by sucrose in strains of Leuconostoc (Funane et al., 1995; Neely & Nott, 1962). No information is available regarding the mechanism of sucrose induction in Lc. mesenteroides. It has been reported (Quirasco et al., 1999) that the dextranxuscrease of Lc. mesenteroides NRRL B-512F is expressed in the presence of carbon sources other than sucrose but at very low levels.

The industrial, food-grade strain Lc. mesenteroides Lcc4 was found to synthesize a dextran which is composed of ζ(1-6)-linked glucose units in the backbone and a minor amount of ζ(1-3) and ζ(1-4) branching (S. Vincent & M. Fischer, personal communication). In this work, the dextranxuscrease of this strain was cloned and sequenced. The protein was characterized and its expression under different fermentation conditions analysed. Furthermore, we showed heterologous expression of the dextranxuscrease in the industrially relevant lactic acid bacterium Lactococcus lactis.

METHODS

Bacterial strains and culture conditions. Leuconostoc mesenteroides Lcc4 was obtained from the Nestlé Culture Collection (Nestlé Research Centre, Lausanne, Switzerland). Esherichia coli SURE (Stratagene) and DH5α (Hanahan, 1983) served as cloning hosts for vector construction and DNA sequencing. Lactococcus lactis MG1363 (Gasson, 1983) was used as heterologous host for expression studies of the Lcc4 dextranxuscrease.

L. mesenteroides Lcc4 was routinely grown at 30°C in MRS (Difco; De Man et al., 1960) and for fermentation purposes at 23°C in Lcc4 medium (K2HPO4 20 g l⁻¹; Difco Bacto-peptone, 5 g l⁻¹; Eridding yeast extract, 5 g l⁻¹; MnSO4.H2O, 0.02 g l⁻¹; NaCl, 0.01 g l⁻¹; FeSO4.7H2O, 0.01 g l⁻¹; MgSO4.7H2O, 0.2 g l⁻¹; pH 6-7 adjusted with H3PO4) supplemented with sucrose (20 g l⁻¹) or glucose (50 g l⁻¹). For inoculation of the fermenter, two precultivation steps were performed. A first preculture was obtained by inoculation of 10 ml MRS with cryoculture and overnight incubation at 30°C without agitation; a second preculture was obtained by inoculation of 200 ml Lcc4 medium in Erlenneyer shake flasks with 1% of the first culture and incubation at 30°C with 9 h with agitation (100 r.p.m.). The main culture was inoculated with 1% (v/v) of the second preculture. The pH-controlled batch and fed-batch cultures of L. mesenteroides were performed in a 15 l MBR bioreactor (New MBR) with a 7 l working volume. Under standard conditions, the fermentation was carried out at 23°C, with an agitation of 200 r.p.m., at an aeration rate of 0.7 l air min⁻¹ and at constant pH value 6-7. For fed-batch fermentations, a pH-neutralizing solution (58.7 g NaOH l⁻¹, 58.7 g KOH l⁻¹) and a concentrated sucrose solution (600 g l⁻¹) were added at an identical rate using two pumps. A pH controller activated both pumps.

L. lactis MG1363 was grown at 30°C without agitation in M17 medium (Difco) supplemented with 5 g glucose l⁻¹ (GM17: Bacto tryptone, 5 g l⁻¹; Bacto Soytone, 5 g l⁻¹; meat digest, 5 g l⁻¹; yeast digest, 2.5 g l⁻¹; ascorbic acid, 0.5 g l⁻¹; MnSO4, 0.25 g l⁻¹; Na2HPO4, 19 g l⁻¹; glucose, 5 g l⁻¹; pH 6.9). pH-controlled fed-batch fermentations of L. lactis pNZDSF were performed as described for Lc. mesenteroides with the following modifications. Under standard conditions, GM17 medium containing sucrose (20 g l⁻¹) and chloramphenicol (5 mg l⁻¹) was used and the cultivation was carried out at 30°C, at an aeration rate of 0.7 l air min⁻¹, with agitation of 200 r.p.m. and at a constant pH of 6.7. The two sterile feeding solutions were a base solution (77 g NaOH l⁻¹, 77 g KOH l⁻¹) and a concentrated glucose solution (600 g l⁻¹). They were added via two pumps activated by the same pH controller. The glucose concentration was kept constant during fermentation using on-line HPLC measurements (as described below) in order to manually adapt the glucose addition rate.

E. coli strains were grown in Luria–Bertani (LB) medium (Difco) while shaking at 37°C.

Plasmids and plasmid constructs. The E. coli vectors pUC18 (Yanisch-Perron et al., 1985) and pBluescriptII KS(+) (Stratagene) were used for cloning DNA fragments for sequencing. For cloning and expression in L. lactis, the vector pNZ124 (Platteeuw et al., 1994) was used. For expression of the Lc. mesenteroides Lcc4 dextranxuscrease dsrD in L. lactis MG1363, the plasmid pNZDSF was constructed, which contains the complete dsrD gene and 499 nt upstream of the predicted start codon as the putative promoter region. The dsrD promoter region together with the entire dsrD gene was amplified by PCR using the primers Prad (5′-GCAAGCTTCAAGACCGTCGTTATATGC-3′) and PostDSII (5′-GCTGAAAGGTGGTCACCATGGACGCCTG-3′) in which restriction sites (the underlined sequences) had been introduced for cloning. The PCR product obtained was cleaved with XbaI and Aval and ligated into the vector pNZ124 previously digested with the same enzymes.

DNA preparation and transformation. Chromosomal DNA from Lc. mesenteroides was isolated according to Marmur (1961). E. coli plasmid DNA was prepared using the Qiagen plasmid mini and maxi kits (Qiagen) according to the protocol supplied by the manufacturer. L. lactis plasmid DNA was prepared essentially as described for E. coli, with the exception that the cells were first treated with lysozyme (5 mg ml⁻¹) for 10 min at 37°C. For the preparation of Lc. mesenteroides plasmid DNA, the cells were first incubated for 1 h at 37°C with Pronase E and proteinase K (at 500 µg ml⁻¹ and 250 µg ml⁻¹, respectively) and then for another 1 h at 37°C with mutanolysin and lysozyme (at 100 µg ml⁻¹ and 10 mg ml⁻¹, respectively). Subsequently, the preparation was continued as described for E. coli. L. lactis and E. coli were transformed by electroporation (Dover et al., 1988; Holo & Nes, 1989).

Molecular techniques. For PCR, Pwo polymerase, the Expand High Fidelity PCR system and the Expand Long Template PCR system, all from Roche, were used. Double-stranded DNA was sequenced by the dideoxy chain-termination procedure, using the thermo sequenase fluorescent-labelled primer cycle sequencing kit (Amersham) and the Li-Cor DNA Sequencer (LICOR). DNA sequences were analysed using the GCG software package (Wisconsin Sequence Analysis Package, Genetics Computer Group). The program BLAST (Altschul et al., 1997) was used for protein similarity searches. Southern blot analyses were performed using the DIG High Prime DNA labelling and detection kit II according to the protocol supplied by the manufacturer (Roche). Other DNA manipulations, gel electrophoresis procedures and molecular techniques followed established protocols (Sambrook et al., 1989).

mRNA manipulations. Total RNA from Lc. mesenteroides cells was isolated using, with a few modifications, the Macaloid clay method
Roche) was used for preparation of an RNA probe. Hybridization Northern blot analyses of the dsrD gene of the L. mesenteroides Lcc4 culture supernatant was mixed with 3 ml substrate buffer (200 mM sucrose, 20 mM sodium acetate, pH 5.2, 20 mM CaCl2, 0.2 g sodium azide 1 ?). A 1 ml sample of this mixture was immediately frozen at −20°C as a control, and 1 ml was incubated for 1 h at 30°C. After incubation, the Nelson test (Nelson, 1944) was performed. As standard solution, 0.6 mM fructose in substrate buffer was used. A standard unit (U) of dextranucrase activity was defined as the amount of enzyme that catalyses the formation of 1 μmol D-fructose min−1 at 30°C.

In situ dextranucrase activity analysis after SDS-PAGE was performed essentially as described by Ferretti et al. (1987). After electrophoresis, the gel was washed overnight without shaking in sodium acetate buffer (20 mM, pH 5.4) containing CaCl2 (0.34 mM) and Triton X-100 (0.1%, v/v). Subsequently, the gel was incubated for 24 h without shaking in the same buffer supplemented with 100 g sucrose l−1. The gel was then fixed for 30 min in 75% ethanol and treated on a shaker for 30 min with 0.7% (w/v) periodic acid in 5% acetic acid. After several washes in 0.2% (w/v) sodium metabisulphite in 5% acetic acid for an additional 60 min, the gel was placed in Schiff reagent (Sigma) until the activity bands appeared, and washed thereafter in 10% methanol/10% acetic acid/80% water. All procedures were carried out at room temperature. The intensities of the active bands of DsrD were quantified using the program Quantity One (Bio-Rad). Increases in the band intensities of the standard were then used to determine the sample activity.

Detection of levan production and levansucrase activity. Lcc4 culture and incubated at 23°C for 24 h. For purification of the exopolysaccharides produced, proteins were precipitated and cells broken open by adding 25 g trichloroacetic acid to 100 ml of the culture. After 1 h stirring at room temperature, the solution was centrifuged at 13700 g for 1 h at 5°C. The water-soluble exopolysaccharide in the supernatant fraction was precipitated by adding 100 ml acetone and stirring overnight at 4°C. After centrifugation (27500 g, 1 h, 4°C), the supernatant was discarded with extreme care. The pellet was washed with 200 ml 50% acetone, and centrifuged again at 27500 g for 1 h at 4°C. The supernatant was again discarded and the residual acetone was evaporated in an open beaker (1 h, room temperature). The pellets were then resuspended in 3% (w/v) NH4HCO3, dialysed against water for 60 h. Finally the samples were lyophilized. For analysis of the monosaccharide composition, 100 μg of the lyophilized sugars were resuspended in 1.5 ml 4 M trifluoroacetic acid and incubated for 1 h at 125°C. After evaporation of the water under nitrogen, 1 ml deionized water was added, and the solution was vortexed and filtered (membrane of 0.2 μm diameter pores). The monosaccharide composition was then determined by HPLC.

For detection of levansucrase activity, an in situ activity assay after SDS-PAGE was performed as described for dextranucrase but with raffinose (100 g l−1) instead of sucrose as a substrate. Increased sample volumes (up to 1 ml culture supernatant after fed-batch fermentation in the presence of sucrose) and incubation times (up to 3 days) were tested.

Analytical determinations. Sucrose, glucose and fructose in the culture supernatant were determined using a Hewlett Packard Series 1100 HPLC fitted with an Aminex HPX 87H column (Bio-Rad) heated to 35°C. Elution was done with 5 mM H2SO4 (flow rate 0.6 ml min−1). Sample volume for injection was 20 μl. Detector for total sugar was a refractometer (HP 1047A). A 5 ml sample of the culture supernatant was run in the presence of either sucrose, glucose or fructose standards from Merck. The compounds were detected with a refractometer (HP 1047A) to detect any contaminating compounds. Quantification of protein concentrations was done as described by Bradford (1976) using bovine serum albumin (1 mg ml−1) as standard.
RESULTS AND DISCUSSION

Isolation and analysis of the dsrD dextranucrase gene from Lc. mesenteroides Lcc4

An alignment of the three Lc. mesenteroides dextranucrase genes reported so far – dsrS from Lc. mesenteroides NRRL B-512F (Wilke-Douglas et al., 1989), and dsrA and dsrB from Lc. mesenteroides NRRL B-1299 (Monchois et al., 1996, 1998) – identified several conserved regions (Fig. 1a). dsrA is significantly shorter than dsrB and dsrS and lacks the 5‘ end encoding the N-terminal part of the protein, including the signal peptide for secretion (Monchois et al., 1996). For a specific amplification of internal portions of the dextranucrase gene from Lc. mesenteroides Lcc4, two sets of degenerate primers, dsrBS1 + dsrABS2 and dsrABS1 + dsrABS2 (Fig. 1a, b) were designed along three conserved regions. Primer dsrBS1 only hybridizes to the longer dextranucrase form.

PCR amplification with the two degenerate primer sets on Lc. mesenteroides Lcc4 genomic DNA resulted in two unique fragments of the expected sizes, 1.5 and 2.4 kbp. This indicated the presence of a certain degree of sequence conservation between the dextranucrase gene of Lc. mesenteroides Lcc4 and the reported ones from the other strains. Subsequently, the 1.5 kbp PCR product was used to probe Lc. mesenteroides genomic DNA digested with various restriction endonucleases. A hybridizing 6 kbp genomic EcoRI fragment was cloned in pBluescriptII KS(+) from a partial library enriched for 4–8 kbp DNA fragments. The correct clone was identified by PCR and by Southern blot analysis. Its insert was sequenced and found to contain a partial ORF with a deduced amino acid sequence similar to the known dextranucrases. A potential ribosome-binding site sequence (AAGGGGA) was found at an appropriate distance from the predicted ATG start codon (Fig. 2). By Southern hybridization analysis, a 5 kbp PsI genomic fragment that overlaps the EcoRI fragment and includes the missing 3‘-end of dsrD was identified (Fig. 1b). The fragment was cloned in pBluescriptII KS(+) and the nucleotide sequence of the ORF was completed.

The identified Lc. mesenteroides Lcc4 dextranucrase gene, 4.6 kbp in length, was named dsrD. It encodes a protein of 1527 aa. The amino acid sequence is highly similar to DsrS (98 % identity). The amino acids different between the two proteins are scattered over the entire sequence. DsrD also shows significant similarities to other dextranucrases such as DsrB (67 % identity) and DsrA (50 % identity). The highly conserved residues identified in the N-terminal domain (Monchois et al., 1999) were also conserved in DsrD. Analysis of the DsrD protein sequence revealed that it consists of a hydrophilic core domain with a hydrophobic N-terminal region. This N-terminal region
displayed the sequence characteristics of a typical signal peptide sequence for secretion, i.e. a core hydrophobic domain flanked by a basic and a more polar region at its up- and downstream sides, respectively.

A putative promoter region was identified upstream of dsrD, as well as a predicted stem–loop structure as potential rho-independent transcription terminator located downstream of dsrD ($\Delta G = -18.9$ kcal mol$^{-1}$; $-79.1$ kJ mol$^{-1}$).

**Characterization of the DsrD protein**

In order to substantiate the assumption that DsrD is translocated through the bacterial cell membrane and secreted into the culture medium, culture supernatant after fermentation of L. mesenteroides in the presence of sucrose was analysed by SDS-PAGE. After electrophoresis, the SDS gel was either stained with Coomassie blue or used for an in vitro assay revealing protein(s) with glucose-polymerizing activity in the presence of sucrose. Several proteins were identified in the supernatant by Coomassie blue staining but only one major protein band, with an estimated size of about 165 kDa, showed dextranucrase activity (data not shown). To confirm that the identified protein band was not a mixture of two or more proteins and furthermore corresponded to the gene product of the sequenced dextranucrase gene, the band was extracted and subjected to N-terminal amino acid sequence analysis. The first six amino acids were identified as D-S-S-V-P-D. This sequence is identical to that of the deduced amino acid sequence of DsrD, starting at position 43 (Fig. 2). Thus, the secreted protein has a calculated molecular mass of 165 kDa.

This allowed, for the first time, determination of the functional signal leader sequence of a dextranucrase enzyme as well as its putative signal peptide cleavage site sequence identified as amino acids V-L-G (Fig. 2). The result is in agreement with that of the ‘−3, −1’ rule proposed by von Heijne (1983) for general signal peptide cleavage sites, according to which the residues at −3 and −1 (relative to the cleavage site) must be small and neutral for cleavage to occur correctly. Furthermore it has been reported that the first few positions downstream of the cleavage site of mature proteins generally show preferences for alanine, negatively charged amino acids (D or E) and hydroxy amino acids (S or T) (Nielsen et al., 1997). The determined N-terminus of DsrD contains such amino acids. However, the DsrD signal peptide is rather long. Computer analysis predicted a cleavage site at position 34 to 36 (A-L-A) resulting in a cleavage between the amino acids at positions 36 and 37 (A-T) (SignalP; Nielsen et al., 1997, 1999). Hence, it cannot be excluded that after an initial signal peptide cleavage, DsrD is further processed to its active form by yet another protease.

In the activity assay, in addition to the major band at 165 kDa, a very faint band at 160 kDa was observed (data not shown). This is probably produced by proteolytic digestion of the original 165 kDa protein. However, no bands at lower molecular masses were detectable even when higher quantities of dextranucrase were loaded on the gel (data not shown). As a control, the culture supernatant of L. mesenteroides Lcc4 was also analysed for levansucrase activity. For this, the in situ gel activity assay was repeated but using raffinose as specific substrate, which cannot be used by dextranucrases. However, no levansucrase activity was detectable even with application of large quantities of supernatant and after several days of incubation. In addition, the exopolysaccharides produced during fermentation were purified and their monosaccharide compositions were determined after hydrolysis of the lyophilized sugars. The only constituent of the exopolysaccharide was glucose. No fructose could be found, confirming that L. mesenteroides Lcc4 did not express detectable levansucrase activity under our fermentation conditions. This is in agreement with the absence of detectable sucrose-polymerizing activity at the lower molecular masses.

For L. mesenteroides strain NRRL B-512F, multiple dextranucrase forms of different molecular masses were observed in SDS-PAGE analysis (Funane et al., 1995; Kobayashi & Matsuda, 1986; Miller et al., 1986; Quirasco et al., 1999). Quirasco et al. (1999) characterized four bands of 170, 160, 116 and 97 kDa with polymerizing activity after SDS-PAGE. Whereas the 170 kDa protein corresponded to the known dextranucrase DsrS of L. mesenteroides NRRL B-512F (Wilke-Douglas et al., 1989), the 160 kDa band was suggested to be produced by proteolytic degradation of the original 170 kDa protein (Sanchez-Gonzalez et al., 1999; Quirasco et al., 1999). The 116 and 97 kDa proteins were identified as levansucrases using the specific in situ gel activity assay with raffinose (Quirasco et al., 1999).

**Mapping of the dsrD promoter**

Primer extension experiments were performed with the synthetic oligonucleotide PE-DS2 and with RNA isolated from L. mesenteroides Lcc4 cells grown in pH-controlled (pH 6.7) fed-batch fermentation on sucrose. The experiments revealed two transcription initiation sites (Fig. 3) located 34 and 35 nt upstream of the predicted start codon of dsrD (Fig. 2). Since the intensity of the two bands was similar, both sites were equally effective in driving transcription initiation. Similar results were obtained with two different primers hybridizing further up- or downstream as compared with PE-DS2 (data not shown). The deduced −10 region (TATAAT) matches the E. coli consensus sequence, whereas the −35 region (CTGTTT) differs somewhat from the optimal consensus sequence for $\sigma^{70}$-dependent promoters.

**Analysis of dsrD expression in Lc. mesenteroides Lcc4**

To analyse induction of dsrD by sucrose and to monitor expression of dsrD during growth, two fermentations were
carried out. First, in a pH-controlled (pH 6 - 7) batch fermentation *Lc. mesenteroides* Lcc4 was cultivated with 50 g glucose l\(^{-1}\) as carbon source to analyse *dsrD* expression in the absence of sucrose. Bacterial growth monitored as OD\(_{590}\), and the dextransucrase activity in the culture were measured (Fig. 4a). Due to the presence of glucose in the culture, which caused a high background in the Nelson assay, the dextransucrase activity was measured by SDS-PAGE and subsequent *in vitro* activity staining. The activity values were then computed using a standard from a sucrose-grown culture of known activity. In parallel, cells were harvested for extraction of total RNA. Samples of the RNA were separated on a formaldehyde gel and dextransucrase-specific mRNA was subsequently detected by Northern blot analysis using a *dsrD*-specific DIG-labelled RNA probe.

A weak band of 4\(-\)6 kbp – corresponding to the expected size of the dextransucrase *dsrD* mRNA – was detected by Northern blot analysis (Fig. 4b). At the same time, low dextransucrase activity could be measured in the culture supernatant and in cell association during fermentation (Fig. 4a).

In a second step, a pH-controlled (pH 6 - 7) fed-batch fermentation was performed on sucrose. By using a sucrose feed during the fermentation, the concentration of the inducer sucrose could be maintained constant at 20 g l\(^{-1}\). This was confirmed by HPLC analysis (data not shown).
shown). Using the Nelson method, dextran sucrose activity in the culture was monitored during growth of *Lc. mesenteroides* Lcc4 (Fig. 5a) and in parallel, cells were used for RNA extraction. As described for the glucose culture, dextran sucrase-specific mRNA was detected by Northern blot analysis using the *dsrD*-specific probe.

It was shown that the *dsrD*-specific 4.6 kbp transcript appeared rapidly after onset of growth (Fig. 5b). With a time delay of 2–3 h, dextran sucrose activity was measured in the culture (Fig. 5a). Whereas the relative concentration of *dsrD* mRNA was maximal after 10–16 h of growth, maximal dextran sucrase activity (of 4 U ml⁻¹) was observed at the end of exponential growth, i.e. after 16 h. In the stationary phase, the dextran sucrose activity decreased slowly although small relative concentrations of transcript were detectable. This indicated that some protein turnover still took place and dextran sucrase was built *de novo* in parallel to the inactivation of a part of the enzyme. Interestingly, the same expression profile (dextran sucrase activity and *dsrD* mRNA) was obtained in a parallel fermentation in which the sucrose feed was stopped at the end of the exponential growth phase (data not shown). The slow decrease in sucrose concentration during stationary phase did not affect the dextran sucrose activity.

*dsrD* transcript levels were 10- to 15-fold higher during fermentation with sucrose compared to that on glucose (Figs 5b and 4b). Similarly, dextran sucrose activity of culture after sucrose fermentation was 10- to 15-fold higher than that measured during glucose fermentation (Figs 5a and 4a). Interestingly, in a fed-batch fermentation with both sugars, glucose and sucrose, production of dextran sucrose activity was similar to that obtained with sucrose alone (up to 4 U ml⁻¹; data not shown). Since glucose metabolism occurred simultaneously with dextran sucrose synthesis, presence of glucose in the medium did not seem to influence dextran sucrose induction by sucrose. Taken together, these results showed that dextran sucrose expression is relatively low in the presence of only glucose and is significantly induced by sucrose.

HPLC analysis revealed that no glucose and only a small concentration of fructose was detectable in the culture medium during fermentation (data not shown). This indicated that fructose was immediately metabolized by *Lc. mesenteroides* Lcc4. Hence, its metabolism differs from that of strain *Lc. mesenteroides* NRRL B-512F, which expresses a dextran sucrase very similar to that of the Lcc4 strain. In *Lc. mesenteroides* NRRL B-512F, as well as in other strains, significant amounts of fructose were released during the first hours of growth on sucrose which were only consumed once sucrose was depleted (Dols *et al.*, 1997; Lawford *et al.*, 1979; Neely & Nott, 1962; Quirasco *et al.*, 1999).

The limited information available about the regulation and expression of dextran sucrases is mainly based on batch fermentation studies performed with or without pH control (Dols *et al.*, 1997; Quirasco *et al.*, 1999) in which the two important parameters, pH and sucrose concentration, may vary. We chose pH-controlled fed-batch fermentations in which the pH and the concentration of the inducer sucrose were maintained constant, to allow expression analysis independent of these parameters. The fermentations were performed at pH 6.7, which was reported as being the best for enzyme production (Alsop, 1983). However, the optimal pH for enzyme activity is 5–5.4 and only limited activity is retained at pH 6.7 (Miller *et al.*, 1986). For this reason viscosity problems due to dextran formation were drastically reduced at pH 6.7, which represented a major advantage for further handling of the culture. A sucrose concentration of 20 g l⁻¹ was sufficient to ensure induction of enzyme synthesis. Higher
concentrations (up to 60 g l\(^{-1}\)) did not result in a further increase of enzyme synthesis (data not shown). The activity produced by \(Lc.\ mesenteroides\) Lcc4 was lower than that reported for \(Lc.\ mesenteroides\) NRRL B-512F, which reached up to 17 U ml\(^{-1}\) during fed-batch culture (Alsop, 1983).

The combination of pH-controlled fed-batch fermentation with Northern blot analysis clearly revealed that transcription of the \(dsrD\) gene was maximal in the exponential growth phase and ceased rapidly upon entry into stationary phase. This directly relates expression of the dextranase gene to the growth of the bacteria. A growth-dependent production of dextranase was suggested earlier for the dextranase \(DsrB\) of \(Lc.\ mesenteroides\) NRRL B-1299 considering specific growth rates and dextranase production (Dols et al., 1997). Similarly for the dextranase \(Dsrs\) of \(Lc.\ mesenteroides\) NRRL B-512F, maximal dextranase activity during batch fermentation with sucrose was observed at the end of the exponential growth phase (Quirasco et al., 1999). With the onset of the stationary phase, the dextranase activity rapidly decreased to less than 15\%.

To verify if host-based regulatory protein(s) possibly present in \(Lc.\ mesenteroides\) but not in a heterologous host, 

\[ \text{Heterologous expression of dextranase} \]

\[ dsrD \text{ in Lactococcus lactis} \]

To express \(dsrD\) in a heterologous host, \(dsrD\) including 499 nt of upstream sequence including the promoter region was amplified by PCR and cloned in vector pNZ124 to result in plasmid pNZDSF. This plasmid was transferred to the non-exopolysaccharide-producing host \(L.\ lactis\) MG1363. The ability of \(L.\ lactis/pNZDSF\) to produce dextran was tested with an agar plate assay in which the strain was grown on GM17 supplemented with 30 g sucrose l\(^{-1}\). From the slimy appearance of the colonies it was evident that the recombinant \(L.\ lactis\) strain produced the dextran. \(L.\ lactis\) containing pNZ124 as a control did not show dextran synthesis. Thereafter, \(dsrD\) expression and dextranase activity were monitored during a pH-controlled fed-batch fermentation using GM17-medium supplemented with 30 g sucrose l\(^{-1}\) and chloramphenicol for plasmid maintenance. Since \(L.\ lactis\) MG1363 is not able to metabolize sucrose (Gasson, 1983), glucose was used as carbon source for the strain and added in the feeding solution. The concentrations of sucrose and glucose during the fermentation were measured on-line by HPLC. The pH was maintained at 6.7. The OD\(\text{_{590}}\) was measured at different time intervals and samples were withdrawn at the same time for dextranase activity measurements (Fig. 6a). The glucose concentration was maintained between 5 and 9 g l\(^{-1}\). The sucrose concentration was constant at 20 g l\(^{-1}\) during the fermentation. Of the presence of glucose in the culture, dextranase activity was measured by SDS-PAGE and activity staining.

![Fig. 6. Time-course of \(dsrD\) expression by \(L.\ lactis/pNZDSF\) during the pH-controlled (pH 6.7) fed-batch fermentation.](image)

One protein migrating at the same molecular mass as observed for Lcc4 (165 kDa) showed dextranase activity both in the culture medium and in the cell fraction (data not shown). The negative control \(L.\ lactis/pNZ124\) did not show any activity in this assay (data not shown). The activity increased towards the end of the exponential growth phase and decreased in the late stationary phase (Fig. 6a). These data demonstrate that the dextranase gene \(dsrD\) of \(Lc.\ mesenteroides\) Lcc4 is expressed and secreted in \(L.\ lactis\) as heterologous host and is able to drive dextran synthesis. However, the dextranase activity produced was significantly lower (fivefold) than that found for \(Lc.\ mesenteroides\).

To verify if host-based regulatory protein(s) possibly present in \(Lc.\ mesenteroides\), but not in a heterologous
host such as L. lactis, are involved in the sucrose induction of dsrD, a pH-controlled fed-batch fermentation was performed with only glucose and no sucrose present. The concentration of glucose during fermentation was kept constant as described above. The growth (OD590) and the concentration of glucose during fermentation was kept performed with only glucose and no sucrose present. The concentration of glucose during fermentation was kept constant as described above. The growth (OD590) and the concentration of glucose during fermentation was kept constant as described above. The growth (OD590) and the concentration of glucose during fermentation was kept constant. Hence, in contrast to the situation in Lc. mesenteroides, the production of dextran-sucrase activity in L. lactis does not change in the presence of sucrose, and hence is not induced by this sugar. This indicates the involvement of Leuconostoc regulatory protein(s) in sucrose induction.

Only limited information is available about expression of dextran-sucrases in heterologous host systems. The dextran-sucrase gene dsrB of Lc. mesenteroides NRRL B-1299 has been cloned and expressed in E. coli (Monchois et al., 1998). Under the control of its original promoter but also with an inducible promoter, the activity of DsrB was more than 1000-fold lower than in Leuconostoc. The activity was measured in the cell fraction after disruption of the E. coli cells. Recently, a gene encoding alternansucrase, a glucosyl-transferase synthesizing a polymer of glucopyranosyl residues from sucrose alternately linked by α(1-6) and α(1-3) osidic bonds in the main chain, was expressed in E. coli (Arguello-Morales et al., 2000). As observed for DsrB, detectable enzyme activity was found in cell extracts after sonication. Heterologous expression in the Gram-positive L. lactis, as presented in this work, yielded a much more efficient dextran-sucrase expression and secretion as compared to the previous work in Gram-negative E. coli. However, the dextran-sucrase activity was still lower in L. lactis in comparison to its natural expression in Lcc4. Since the fermentation conditions for L. lactis and Lc. mesenteroides were rather similar and the enzyme is sufficiently stable under these conditions, the differences in activity are most likely the result of lower expression in L. lactis, probably due to lower promoter activity of the recombinant dsrD construction in L. lactis. It can be speculated that higher expression levels may be achieved by selecting appropriate L. lactis promoter constructs for expressing the dsrD gene.

Leuconostoc strains produce CO2 upon fermentation, which seriously limits their industrial applicability for production of fresh dairy products, such as yoghurt and fresh cheese-type products. Hence, to make use of the texturing properties of the dextran-sucrase, the enzyme has to be isolated and added to a milk fermentation process. Our results on the expression of this enzyme in fed-batch fermentations may help to optimize the industrial process of dextran-sucrase production. Our results further demonstrate that a dextran-sucrase can also be efficiently expressed and secreted in a non-Leuconostoc, heterologous food-grade lactic acid bacterium. This opens interesting new possibilities for industrial applications of dextran-sucrases, produced in situ, during the food fermentation process. This may bring new opportunities to the dairy industry to improve the texture quality of fresh fermented dairy products.

ACKNOWLEDGEMENTS

We are indebted to Stéphane Duboux, Michel Richard and Yann Hautier for expert technical assistance. We thank Philippe Duboc for critically reading the manuscript.

REFERENCES


