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Increased Exopolysaccharide Production in *Lactococcus lactis* due to Increased Levels of Expression of the NIZO B40 *eps* Gene Cluster

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Exopolysaccharides (EPS) play an important role in the rheology and texture of fermented food products. This is the first report demonstrating that homologous overexpression of a complete *eps* gene cluster in *Lactococcus lactis* leads to increased EPS production levels. A ninefold-elevated EPS plasmid copy number led to an almost threefold increase in the *eps* expression level, resulting in an almost fourfold increase in the NIZO B40 EPS production level. It was previously reported that increased EPS precursor levels did not influence NIZO B40 EPS production levels. However, the present results indicate that the maximal NIZO B40 EPS production level is limited by the activity level of the expression products of the *eps* gene cluster rather than by the level of EPS precursors.

In the dairy industry, exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) are used to improve the texture of fermented diary products. Different LAB produce a wide variety of EPS that have potential applications as food additives. These EPS would be preferable over presently used stabilizers, such as xanthan, since their production hosts have a food-grade status. However, LAB EPS are formed only at relatively low production levels (40 to 800 mg liter^{-1}) compared to those of commercially produced EPS (10 to 25 g of xanthan liter^{-1}) (1). Nevertheless, some EPS produced by LAB are very effective biothickeners when produced in situ (5, 13). Among EPS-producing LAB, one of the best characterized is *Lactococcus lactis* NIZO B40, which harbors a 42,180-bp EPS plasmid, pNZ4000, containing the 12-kb *eps* operon (14, 16). Previously it was demonstrated that the overproduction of the NIZO B40 priming glucosyltransferase resulted in a 15% increased EPS production compared to that of the control strain (15). These data suggest that elevation of the level of *eps* gene expression could result in higher EPS production.

To elevate the level of *eps* gene expression, we cloned the entire NIZO B40 *eps* gene cluster of pNZ4000 (14) on the high-copy-number vector pIL253 (10), yielding pNZ4120. This was achieved by introducing an *Nco*I site with the help of a double-stranded oligonucleotide (link-F and link-R; Table 1) that was subsequently used for the cloning of the 17-kb *Nco*I fragment of pNZ4000, encompassing the entire *eps* gene cluster. Plasmid pNZ4120 was transformed into *L. lactis* NZ9000 (7), and the resulting strain was used to determine the relative copy number of the EPS plasmid (relative to the chromosomal DNA copy number) by using real-time PCR. Exponentially grown lactococcal cells (20 μl) were harvested by centrifugation and were disrupted by a microwave treatment (2 min, 800 W). Disrupted cell pellets, including total DNA, were suspended in 20 μl of water and were directly used for PCRs. These reactions contained the primer pairs designed on *epsC* (TM-epsC-F and TM-epsC-R), *pepN* (TM-pepN-F and TM-pepN-R), and *ery* (TM-ery-F and TM-ery-R) (Table 1) and were performed by using the TaqMan core reagent kit (Applied Biosystems, Nieuwerkerk aan de Ijssel, The Netherlands). The threshold cycle number (C_t) was determined (6) by using the ABI Prism 7700 sequence detection system software and was used to calculate the relative gene copy number (N_{relative}) for each strain with the formula $N_{\text{relative}} = 2^{(Ct\text{gene}X - Ct\ pepN)}$ (Table 2), with the chromosomally carried *pepN* gene as an internal standard of chromosomal DNA quantification. An almost ninefold higher relative EPS plasmid copy number was found in cells of strain NZ9000 harboring pNZ4120 compared to that of cells harboring the pNZ4030 plasmid, indicating that replacing its endogenous replication machinery by that of pIL253 could increase the EPS plasmid copy number significantly.

The effect of increased EPS plasmid copy number on the expression of the *eps* genes was analyzed by quantification of the relative *eps* mRNA level. Therefore, mRNA isolated from exponentially grown cells with the help of an RNeasy kit (Qiagen, Leusden, The Netherlands) was reverse transcribed by using Omniscript reverse transcriptase (Qiagen) and reverse transcriptase primers, which were designed on *epsC* (TM-epsC-RT) or *pepN* (TM-pepN-RT) containing a dedicated 5′ tag for cDNA/DNA discrimination (12) (Table 1). The cDNA generated was subsequently amplified by real-time PCR using the primer pairs designed on *epsC* (TM-epsC-F and TM-epsC-R2) and *pepN* (TM-pepN-F and TM-pepN-R2). The relative expression levels of the *eps* genes in strain NZ9000 harboring pNZ4120 (relative to the expression level of the chromosomally located *pepN* gene) were almost threefold higher than those observed in the same strain harboring pNZ4000, establishing that the expression of the *eps* genes can be raised to a higher level by increasing the copy number of the EPS plasmid.

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TABLE 1. Strain, plasmids, and oligonucleotide sequences of a DNA linker, forward (F) and reverse (R) primers, and probes which contain a G-carboxyfluorescein (FAM) reporter dye and a G-carboxytetramethylrhodamine (TAMRA) quencher dye used in real-time PCR and reverse transcriptase (RT) primers used in RT-PCR

Strain, plasmid, or primer[a]	Relevant characteristics[b]	Source or reference
Strain		
NZ9000	MG1363 *pepN*::*nisRK*	7
Plasmids		
pIL253	Em^r, cloning vector	10
pNZ4000	NIZO B40 EPS plasmid	14
pNZ4030	Em^r, pNZ4000 derivative	14
pNZ4120	Em^r, pIL253 derivative containing a 17-kb *Nco*I fragment carrying the NIZO B40 *eps* gene cluster	This study
Primers		
link-F	5′-TCGAGCATGCCATGGCATGG-3′	
link-R	5′-GATCCCATGCCATGGCATGC-3′	
TM-epsC-F	5′-CATCTTAAATGCACGTGACGT-3′	
TM-epsC-R	5′-AGTGTCACTGGTCATTTTGG-3′	
TM-epsC-R2	5′-ACTTTCATGGATTTGGAAGTGTC-3′	
TM-pepN-F	5′-TTGGCACACAGTTTGAAAGCC-3′	
TM-pepN-R	5′-CAAATCGAAAGTTGCTTTCGC-3′	
TM-pepN-R2	5′-CACTATGGCTAACCGTTAATCG-3′	
TM-ery-F	5′-TTCACCGAACACTAGGGTTGC-3′	
TM-ery-R	5′-CATTCCGCTGGCAGCTTAAG-3′	
TM-epsC-FAM	5′-FAM-CGCATCTGATGCAACAAAATGCGTA-TAMRA-3′	
TM-pepN-FAM	5′-FAM-TTTTGCTCGCCAAGCTTTCCCATCT-TAMRA-3′	
TM-ery-FAM	5′-FAM-TGCACACTCAAGTCTCGATTCAGCA-TAMRA-3′	
TM-pepN-RT	5′-CACTATGGCTAACCGTTAATCGAAAGTTGC-3′	
TM-epsC-RT	5′-ACTTTCATGGATTTGGAAGTGTCACTGGTC-3′	

[a] Primers were purchased from Pharmacia; labeled (FAM, TAMRA) primers were purchased from Applied Biosystems.
[b] Em^r, erythromycin resistant.

Overexpression of the entire *eps* gene cluster resulted in a significantly reduced growth rate and a lower final optical density (Table 2). In addition, the relative carbon flux towards EPS production was threefold increased (Table 3), suggesting that increased EPS production generates a significant metabolic burden due to the required high-level production of sugar nucleotides, which are utilized in both EPS production and growth. This suggestion is supported by the observation that sugar-nucleotide pools in EPS-producing cells are lower than those of non-EPS-producing cells (8, 9).

Previously it was reported that increasing the enzyme activity levels of the household genes involved in the EPS biosynthesis pathway led to increased EPS precursor levels (2, 3). However, this did not result in increased NIZO B40 EPS production levels. In contrast, EPS production was fourfold elevated in the *eps* overexpression strain (Table 2), suggesting that the EPS production level could be directly correlated to the *eps* gene expression level. This would indicate that the maximal NIZO B40 EPS production level is limited by the activity level of the expression products of the *eps* gene cluster rather than by the level of sugar nucleotides. However, this hypothesis might not hold true for the biosynthesis of EPS other than NIZO B40. The latter is supported by the findings that the level of *Streptococcus pneumoniae* type 3 capsular polysaccharide production in *L. lactis* can be dramatically increased by the expression of the pneumococcal capsular polysaccharide precursor-forming enzyme UDP-glucose pyrophosphorylase (4).

To evaluate the effect of EPS overproduction on the biophysical properties of the fermentation broth, the kinetic viscosity of the EPS-overproducing strain was measured by using an Ubbelohde viscometer with a capillary diameter of 0.63 mm (17), and it appeared to be 1.6-fold increased relative to that of the cells producing the native EPS production level (Table 2). These results indicate that improvement of EPS production levels positively influences the viscosity properties of the fermented product.

Here we have described the targeted analysis of an impor-

TABLE 2. EPS production, DNA copy numbers of the NIZO B40 EPS plasmid, and expression levels of the *eps* genes of *L. lactis* strain NZ9000 harboring pNZ4030 or pNZ4120[c]

Strain (plasmid)	Growth rate (h^{-1})	Final OD_{600}[d]	Relative EPS plasmid copy number[a]		Relative *eps* transcription level[a]	EPS[a] level		Kinetic viscosity[b] ($m^2\ s^{-1} \times 10^6$)
			Ery probe	EpsC probe		(mg $liter^{-1}$)	(mg liter $\times$ OD_{600}^{-1})	
NZ9000 (pNZ4030)	0.85 ± 0.003	2.7 ± 0.02	2.2 ± 1.6	1.3 ± 0.1	0.07 ± 0.03	93 ± 7	35 ± 3	1.4 ± 0.1
NZ9000 (pNZ4120)	0.69 ± 0.003	2.4 ± 0.05	16 ± 2.1	13 ± 1.8	0.18 ± 0.01	343 ± 5	128 ± 4	2.2 ± 0.1

[a] Values are averages based on at least three independent experiments.
[b] Values are averages based on at least two independent experiments. The kinetic viscosity of the medium was $1.2 \times 10^6\ m^2\ s^{-1}$.
[c] The EPS plasmid copy number and *eps* expression levels were determined relative to the chromosomally located *pepN* gene copy number and expression level.
[d] OD_{600}, optical density at 600 nm.

TABLE 3. Carbon balance of glucose consumption and product formation in *L. lactis* strain NZ9000 harboring EPS plasmid pNZ4030 or pNZ4120

Strain (plasmid)	Glucose consumption[a] (C mol)	Carbon formation (C mol)[c]				Recovery (%)
		EPS[b]	Lactate[a]	Acetate[a]	Ethanol[a]	
NZ9000 (pNZ4030)	35.8 ± 0.09	0.23 ± 0.01 (0.6%)	30.6 ± 0.02 (85%)	0.21 ± 0.01 (0.6%)	0.03 ± 0.02 (0.04%)	92 ± 0.6
NZ9000 (pNZ4120)	35.1 ± 0.04	0.73 ± 0.04 (2.0%)	27.3 ± 0.28 (78%)	0.33 ± 0.04 (0.9%)	0.33 ± 0.06 (0.9%)	100 ± 2.2

[a] Concentrations of glucose, lactate, acetate, and ethanol in supernatant were analyzed by high-performance liquid chromatography as described by Starrenburg and Hugenholtz (11).
[b] EPS were isolated and analyzed as described previously (8).
[c] The percentages of product formation toward glucose are given in parentheses.

tant bottleneck in EPS production. We could increase the NIZO B40 EPS production level fourfold by overexpression of the NIZO B40 *eps* genes in *L. lactis*. Furthermore, the results suggest that the EPS production level is directly correlated to the *eps* gene expression level and could possibly be raised even further. The identification of EPS production bottlenecks is important for future challenges for the construction of lactococcal strains that produce EPS with novel properties. The results presented here are a first step toward the development of lactococcal production hosts of EPS that could be applied as food additives.

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