**Hansenula polymorpha** maltase gene promoter with sigma 70-like elements is feasible for *Escherichia coli*-based biotechnological applications: Expression of three genomic levansucrase genes of *Pseudomonas syringae* pv. tomato

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Abstract

P_MAL1, the sigma 70-like sequences possessing promoter of the maltase gene of *Hansenula polymorpha*, was evaluated for its application in heterologous protein production in *Escherichia coli*. Levansucrases Lsc1, Lsc2 and Lsc3 of *Pseudomonas syringae* pv. tomato DC3000 were expressed from P_MAL1 in *E. coli* as biotechnologically relevant model proteins. Production of soluble levansucrases with high specific activity confirmed appropriate strength of P_MAL1 in a prokaryotic host. As about 90% of levansucrase activity was present in the cytoplasm of *E. coli*, no levan-synthesis related sucrose intolerance of bacteria was observed. All three levansucrases hydrolyzed and polymerized both, sucrose and raffinose. The raffinose-related activity of levansucrases has not been previously described in *P. syringae*. The P_MAL1 expression system was used to produce Lsc3 protein in *E. coli* for purification. The purified levansucrase showed much higher affinity for sucrose cleavage ($K_m = 21$ mM) than the levansucrase from *P. syringae* pv. phaseolicola described in the literature with $K_m$ of 160 mM.

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**Keywords:** Heterologous protein expression; Sigma 70; Levansucrase; *Pseudomonas syringae*; *Hansenula polymorpha*; Yeast promoter

1. Introduction

In our previous work, we have described the MAL gene cluster that is responsible for the utilization of maltose and sucrose in *Hansenula polymorpha* [1,2]. Cloning of the maltase gene (*HpMAL1*) of this gene was also functional in *Escherichia coli*, as high maltase activity in *HpMAL1*-possessing transformants was observed [3]. Since *E. coli* is the most popular host in recombinant protein production [4], P_MAL1 may find application in biotechnology. To evaluate feasibility of P_MAL1 for *E. coli*-based biotechnological expression systems, levansucrases were chosen as test proteins expressed from the promoter. Levansucrases (EC 2.4.1.10) belong to glycoside hydrolase family GH68 ([http://www.cazy.org/fam/GH68.html](http://www.cazy.org/fam/GH68.html)). They polymerize fructose residues of sucrose to $\beta$-(2,6)-linked fructan polymers (levans) and are considered as proteins of biotechnological interest, because (i) levan polymer has antitumor and anti-obesity activities [5,6]; (ii) levan may be used as emulsifier or encapsulating agent in industry [7]; (iii) besides levan, several levansucrases also produce a considerable amount of prebiotic fructo-oligosaccharides [8–12]. Levansucrases are present in several bacteria with those of *Gluconacetobacter diazotrophicus* [13], *Zymomonas mobilis* [14] and *Bacillus subtilis* [15] studied most thoroughly. Bacterial genomes usually encode a single levansucrase protein whereas a plant pathogen *Pseudomonas syringae* pv. glycinea has three levansucrase genes [16]. Some of these loci may encode proteins with specific functions and novel properties. Up to now, levansucrases of *P. syringae* have been mostly studied from the aspect of bacterial physiology and plant pathogenesis [16,17], and only one levansucrase protein of this species has been purified and thoroughly characterized [18].

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specifcity of Lsc proteins was studied and shown to be different from that of _P. syringae_ pv. phaseolicola levansucrase.

2. Materials and methods

2.1. Strains and plasmids

_P. syringae_ pv. tomato DC3000 ( _P. syringae_ DC3000) was provided by Dr. A. Tover (University of Tartu, Estonia). _E. coli_ strains used in this study were HB101 (lac+), a spontaneous LacY1 revertant of _E. coli_ HB101 (supE44 hsdS20 rK-mac recA13 ara-14 proA2 leuB6 lys25 xyl-5 mtl-1 galK2 lacY1) [19]. RA11tr (Δlac21 melA recA2) [20,21] kindly donated by Dr. J.-C. Paard (Inra, France), and DH5α (Stratagene, CA, USA). pBluescript SK (Stratagene) was used as a source of β-lactamase gene in cell fractionation, pS1 [3] contains the maltase gene HpMAL1 and its full-length promoter in pYT3 [22], Whereas in pS1Mun1 and p5HindIII-Mun1 the promoter was truncated till Mun1 and HindIII site, respectively. pPHPX5 phospho-Spel-Smal has the maltase gene HpMAL1 behind its full-length promoter in pHPX8 [8]. pXM4 MAL1 and pPHPX8 MAL1 contain the maltase reporter gene behind either the methanol oxidase gene promoter in pX4-HNBESX [1] or the TEF2 gene promoter in pHPX8. TEF2 (AY179869) codes for translation elongation factor 1-alpha.

2.2. Construction of lsc expression vectors and transformation

The expression vector pHIPMal harboring the maltase gene ( _pMal_ ) was designed as follows: PMAL was amplified from pS425 - pS1 Spel-Smal [1] with primers MalpromBamHI (Fw) and reverse (Rev) primers (BamHI and SalI restriction sites used for cloning underlined) were as follows: Lsc1FwBam (5′ cagatgcagcagcagcaggg 3′) and Reverse (5′ cagagacacttagctg 3′), and inserted after cleavage with BamHI and NotI into pHPX8, thus replacing the TEF2 promoter by pMAL-L.

The coding regions of _lsc1_, _lsc2_ and _lsc3_ with preceding Shine-Dalgarno sequences and ∼100 bp of 3′-noncoding sequences were PCR-amplified from lysed cells of _P. syringae_ DC3000 using a Pfu DNA polymerase (Fermentas, Lithuania) according to manufacturer’s recommendations. Lsc-specific forward (Fw) and reverse (Rev) primers (BamHI and Sall restriction sites used for cloning underlined) were as follows: Lsc1FwBam (5′ tcggctgcagtccggactgg 3′) and Lsc2Rev (5′ tcggctgcagtccggactgg 3′); Lsc3FwBam (5′ tcggctgcagtccggactgg 3′) and Lsc3RevSal (5′ tcggctgcagtccggactggatg). The amplification products of _lsc1_ and _lsc3_ were cloned with BamHI and Sall and inserted between the same sites of pHIPMal. The Small restriction site of pHIPMal was used for _lsc2_ insertion. The resulting plasmids were designed pHIPMalprom-lsc1, pHIPMalprom-lsc2 and pHIPMalprom-lsc3, respectively. Standard methods of DNA manipulation were used [23]. DNA fragments further used for cloning were gel-purified using Ultra Clean™ 15 kit (Mo Bio, CA, USA). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer’s (Fermentas) recommendations. Bacterial plasmid DNA was purified using Perfectprep plasmid minikits (Eppendorf, Germany). _E. coli_ was transformed with plasmid DNA essentially as described in [3].

2.3. Growth media and cultivation of bacteria

Bacteria were routinely grown in LB medium supplied with antibiotics (kanamycin, 50 μg/ml or ampicillin, 100 μg/ml) to maintain the plasmids. LB-kanamycin agar containing 10% of either sucrose or raffinose was used to detect levansucrase formation by recombinant bacteria. Growth temperature was 37 °C, if not indicated otherwise. Liquid cultures were aerated on a shaker. Growth of _lsc3_-expressing _E. coli_ on sucrose (0.5%) and glucose (0.5%) was inspected in liquid minimal M9 medium [24] supplemented with 10 μg/ml thiamine, 100 μg/ml L-leucine and 50 μg/ml kanamycin. The media were inoculated from overnight cultures of LB-grown cells at initial optical density of culture at 600 nm (∝0.1) and growth curves were obtained.

2.4. Fractionation of cells and preparation of extracts

Periplasmic and cytoplasmic fractions of recombinant _E. coli_ were prepared using osmotic shock treatment [25], but sucrose was replaced with sorbitol to eliminate interference with levansucrase assay. A periplasmic marker (β-lactamase) for the assay was provided by fractionation of a 1:1 mixture of two different recombinant cultures of _E. coli_ HB101 (lac-). The cultures carried pHIPMalprom-lsc3 encoding the Lsc3 protein and pBluescript SK encoding the _E. coli_ β-lactamase, respectively. The cellular fractions were assayed for the activities of levansucrase, β-lactamase and glucose-6-phosphate dehydrogenase, the latter serving as a cytosolic marker.

For preparation of cell extract (it was also used as a fraction of soluble cell proteins), the bacteria were harvested by centrifugation, washed twice in appropriate buffer, sonicated, and the supernatant from centrifugation (20 min, 13 700 g, 4 °C) was used as cell extract. The resulting pellet was treated according to Sunita et al. [26] to isolate the fraction of insoluble proteins.

2.5. Enzyme assay

Levansucrase activity on sucrose and raffinose was determined at 37 °C by monitoring the release of glucose or reducing sugar, respectively. Reaction mixtures (1 ml) contained McIlvaine’s buffer (pH 6.0) with 0.015% Na-azide, sucrose or raffinose, and appropriately diluted enzyme preparation. If glucose release was measured, samples (50 μl) withdrawn at regular intervals were mixed with 150 μl of 200 mM Tris-buffer (pH 8.3), and heated at 96 °C for 5 min. Glucose content of the samples was determined with Glucose Liquecoulor kit (Human GmbH, Germany) according to manufacturer’s instructions. Release of reducing sugar was quantified using a 3,5-dinitrosalicylic acid (DNS) method as follows. Samples (200 μl) withdrawn from the levansucrase assay mixture were combined with 400 μl of DNS reactive [27], heated at 100 °C for 5 min, cooled on ice, 800 μl of distilled water was added and absorption was measured at 540 nm.

Kinetic parameters of the purified Lsc3 protein for sucrose hydrolysis were obtained by monitoring the release of glucose from 5 to 300 mM sucrose in the absence and presence of 100 mM raffinose that was used as an inhibitor. The _Km_ for Lsc3 for raffinose was assayed similarly, but release of reducing sugar from raffinose was measured in a DNS assay. The initial velocities of the reactions were fitted with Enzyme Kinetics Module 1.1 of the Sigma Plot 2001 (SYSTAT) according to Michaelis-Menten equation.

Levansucrase activity was expressed in μmolms of glucose (or reducing sugar in case of DNS assay) produced per min per mg of protein (U/mg). Maltase activity was measured with a chromogenic substrate p-nitrophenyl-α-D-glucopyranoside [3]. β-Lactamase was assayed using benzylpenicillin [28], glucose-6-phosphate dehydrogenase as in Clifton et al. [29], and protein concentration according to Lowry.

2.6. Thin layer chromatography

Thin layer chromatography (TLC) was performed on silica gel 60 plates (Merck, Germany). The levansucrase reaction with purified Lsc protein (~5 μg protein in 1 ml reaction mixture) was conducted as described in Section 2.5 at varied concentrations (10, 20, 50 and 100 mM) of sucrose or raffinose. At selected time points, the reaction was stopped by heating and aliquots (1 μl) were spotted onto a TLC plate alongside with 0.5 μl of 0.1 M reference sugars. The chromatogram was developed twice with a solvent system of chloroform-methanol—water (90:65:15, v/v/v) [30]. The sugars were detected by dipping the plate into a solution of 3% (w/v) urea, 1 M phosphoric acid in water-saturated butanol and subsequent heating at 120 °C for 10 min as in [31].

2.7. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was conducted as described in [23]. Relative amount of Lsc3 protein among soluble proteins of recombinant _E. coli_ was evaluated in a denaturing PAGE assay. For that, varied amounts of cell extract protein from _E. coli_ HB101 (lac+) carrying pHIPMalprom-lsc3 were electrophoresed alongside with different quantities of protein size marker PageRuler™ SM0671 (Fermentas). The intensity of the levansucrase band was compared with those of the PageRuler after staining of the gel with Coomassie brilliant blue. Denaturing PAGE was also used for screening of soluble and insoluble protein fractions of _E. coli_ carrying pHIPMalprom-lsc3 for the
presence of levansucrase band. Respective fractions of E. coli transformed with the empty vector, were used as a control.

To visualize the activity of Lsc1, Lsc2 and Lsc3 proteins with sucrose and raffinose, cell extracts of E. coli transformants producing these proteins were electrophoresed in three parallel gels. One gel was stained for protein with Coomassie brilliant blue, other two were incubated in McIlvaine’s buffer with 10% sucrose or 10% raffinose to identify levan-synthesising bands.

2.8. Protein purification

Lsc3 protein was purified from sonicated extracts of E. coli HB101 (lac+) harboring pHIPMalprom-lsc3, using precipitation of proteins with (NH4)2SO4 and subsequent size-exclusion chromatography on a Sephacryl S-300 column [32]. A detailed procedure of protein purification as well as a thorough characterization of the protein will be published elsewhere.

2.9. Computer databases and programs used

CAZY (http://www.cazy.org/fam/GH08.html), NCBI (http://www.ncbi.nlm.nih.gov/) and P. syringae (http://www.pseudomonas-syringae.org/) databases were used for analysis of the genomes and retrieving the sequences. Proteins were aligned using Clustal W program [33]. Molecular weights of levansucrase proteins were calculated using Compute pl/Mw tool provided by the ExPasy Proteomics Server (http://www.expasy.org/). Presence of signal peptide in the proteins was predicted with the Signal P 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) [34].

3. Results and discussion

3.1. Evaluation of the performance of yeast promoters in E. coli

We have previously shown that E. coli transformants harboring the H. polymorpha maltase gene (HpMAL1; AL432586) with its native promoter (PMAL1) exhibited a high maltase activity. Thus, PMAL1 was functional in a prokaryotic host [3]. According to the literature, the promoters of some other genes of methylotrophic yeasts (LEU2 of Candida boidinii, URA3 of C. boidinii and H. polymorpha) also function in E. coli, since these genes have been cloned by complementation of respective E. coli mutants [35–37]. In LEU2 promoter, sigma 70-like hexamers were assumed to serve as a promoter in E. coli, albeit no experimental proof was provided [36]. Interestingly, sigma 70-like boxes were also found in PMAL1 [3]. Here, we inspected PMAL1 and two reference promoters (PMOX1 and PTEF2) of H. polymorpha for the expression in E. coli to verify the significance of these sigma 70-like boxes. H. polymorpha maltase gene was used as a reporter since E. coli lacks endogenous maltase activity and a simple chromogenic maltase assay is available [3]. It should be noted that PMOX1 (the promoter of the methanol oxidase gene), is one of the strongest promoters in yeasts. It is very highly induced in methanol-growing H. polymorpha, and has widely been used for recombinant protein production [38]. The reporter plasmids were introduced into E. coli, and performance of the promoters was evaluated according to maltase activity of the transformants. Table 1 shows that PTEF2 and PMOX1 did not function in E. coli; maltase expression from these promoters was only marginal. Consistent with experimental data, sequence analysis of PTEF2 and PMOX1 revealed no sigma 70-like hexamers and Shine-Dalgarno sequences. At the same time, expression of maltase from PMAL1 in E. coli was high in both plasmid backgounds studied. As previously reported by us, PMAL1 possesses sigma 70-like –10 and –35 hexamers at positions –310 to –282 (TTGACA-N17-TATAAT), and –213 to –185 (GGA-TACA-N17-TATAAT) relative to the ATG codon of the maltase gene [1], positions agreeing with the sigma 70 consensus TTGACA-N17-TATAAT [4] are highlighted. A reporter assay of truncated versions of PMAL1 supported functional significance of these sigma 70-like boxes for gene expression in E. coli: shortening of PMAL1 till Munl cleavage site at –315 bp retaining sigma 70-like elements, did not affect maltase activity of the transformants, whereas further truncation till the HindIII cleavage site (eliminating the sigma 70-like hexamers), strongly reduced the activity (Table 1). It should be noted that similarly to PTEF2 and PMOX1, PMAL1 has no Shine-Dalgarno like sequence.

3.2. P. syringae pathovars have multiple levansucrase genes

We chose levansucrases as biotechnologically important test proteins to evaluate PMAL1-directed expression system in E. coli. According to the literature, P. syringae pv. glycinea has...
very low affinity for sucrose cleavage (substrate and is exceptional among levansucrases because of biotechnologically valuable properties, e.g. perfect stability it should belong to a group of 431-aa levansucrases. The P. has been biochemically characterized—Hettwer et al. [18] has AGNINYEPTVWSRADALKVN shown for this protein [18], pv. phaseolicola. According to the sequence fragment purified an extracellular levansucrase protein from P. syringae 3.5). Up to now, only one levansucrase protein of probably they do not function as secretion leaders (see Section extensions of 431 aa levansucrases is not known, but most according to the Clustal W analysis. The role of N-terminal have N-terminal extensions of 16 aa lacking in 415 aa proteins Lactobacillus sanfranciscensis (879 aa) and L. reuteri (804 aa) [39,40]. The 424 aa levansucrase of a Gram-positive bacterium Leuconostoc mesenteroides could be considered exceptional because of its relatively small size and a high similarity to levansucrases from Gram-negative bacteria [12]. Interestingly, the 431 and 415 aa levansucrases of P. syringae differ mostly in their N-termini: the 431 aa proteins have N-terminal extensions of 16 aa lacking in 415 aa proteins (Fig. 1), otherwise they share a high (89–99%) level of identity according to the Clustal W analysis. The role of N-terminal extensions of 431 aa levansucrases is not known, but most probably they do not function as secretion leaders (see Section 3.5). Up to now, only one levansucrase protein of P. syringae has been biochemically characterized—Hettwer et al. [18] has purified an extracellular levansucrase protein from P. syringae pv. phaseolicola. According to the sequence fragment AGNINYEPTVWSRADALKVN shown for this protein [18], it should belong to a group of 431-aa levansucrases. The P. syringae pv. phaseolicola levansucrase exhibited several biotechnologically valuable properties, e.g. perfect stability and toleration of denaturants. It cannot use raffinose as substrate and is exceptional among levansucrases because of very low affinity for sucrose cleavage ($K_m = 160 \text{mM}$) [18].

3.3. Cloning and expression of levansucrase genes of P. syringae pv. tomato DC3000

E. coli is a perfect host for cloning and expression of lsc genes: the laboratory strains of E. coli do not metabolize sucrose [41] and therefore the lsc-expressing transformants will have a dominant sucrose-positive phenotype. The fact that levansucrases of other pathovars of P. syringae were not expressed from their own promoters in E. coli [16,42], justifies application of a heterologous promoter, P$_{MAL}$, in our case. lsc1, lsc2 and lsc3 genes of P. syringae pv. syringae DC3000 (P. syringae DC3000) were cloned into pHIPMalprom under control of P$_{MAL}$. E. coli HB101 (lac+), transformed with resulting plasmids, pHIPMalprom-lsc1, pHIPMalprom-lsc2 and pHIPMalprom-lsc3, formed mucoid colonies on LB-kanamycin plates containing 10% sucrose, revealing synthesis of functional levansucrases whereas control transformants with empty vector pHIPMalprom, had a nonmucoid phenotype. The exopolysaccharide produced by lsc-expressing clones in LB-sucrose medium, was hydrolyzed and subjected to biochemical analysis. Expectedly, the polymer had a 99:1 ratio of fructose to glucose typical for levan (data not shown).

Table 2 presents levansucrase activities due to the expression of lsc1, lsc2 and lsc3 in two different strains of E. coli. The highest levansucrase activity was recorded for lsc3 in E. coli HB101 (lac+), and the lowest for lsc2 in E. coli RA11r.

Due to levansucrase activity, recombinant E. coli gained the ability to grow on sucrose minimal medium. Fig. 2 shows that the lsc3-expressing E. coli grows on sucrose and glucose equally well given that mostly glucose moiety of sucrose can be used for growth as fructose is partly built into the levan polymer.

3.4. Levansucrases are prominent proteins in cell extracts of recombinant E. coli

Proteins in cell extracts of lsc-expressing E. coli were separated by non-denaturing PAGE and stained in parallel for protein and levansucrase activity (Fig. 3). In approximately 30 min of incubation, whitish levan bands were clearly visible in sucrose-immersed gel whereas synthesis of levan from raffinose was slower, it was evident after about 90 min of incubation with raffinose. Raffinose utilization by levansucrases will be addressed in a more detail in Section 3.6. Comparison of the gels shows that levan-synthesizing bands correspond to most prominent protein bands of the reference gel shown in Fig. 3A. Lsc2 protein that is smallest among of three
levansucrase proteins (415 aa, calculated molecular weight 45.8 kDa), migrated in the gel most rapidly. The calculated molecular weights of Lsc1 and Lsc3 proteins (both 431 aa long) were 47.6 and 47.7 kDa, respectively. The PAGE shows that despite Lsc1 and Lsc3 have quite similar calculated sizes, they had slightly different mobility.

Denaturating PAGE was run to evaluate relative amount of Lsc3 protein among total soluble proteins of host bacteria (see Section 2.7). Lsc3 was calculated to comprise about 20% of total soluble proteins of *E. coli*. That level of expression is considered optimal for *E. coli* production systems [4]. On the other hand, a high expression level may result in production of insoluble aggregated protein. For example, levansucrase of *Z. mobilis* (LevU) was produced in *E. coli* at a high level (20% of total cellular proteins) if a constitutive promoter of *Rahnella aquatilis* was used and bacteria were grown in fed-batch culture, but the protein accumulated as inclusion bodies and required renaturation. In shake flask culture, the yield of levansucrase was lower, but it was produced as active and soluble protein [26]. A widely used strong and inducible expression system based on T7 RNA polymerase also resulted in rapid inactivation of LevU protein and formation of inclusion bodies in *E. coli* [43].

To rule out the possibility of levansucrase inactivation in case of our expression system, a denaturating PAGE of soluble levansucrase activity of *Escherichia coli* strains HB101 (lac+) and RA11r* harboring the expression plasmids with lsc genes of *P. syringae pv. tomato* DC3000

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Plasmid*</th>
<th>Lsc gene expressed</th>
<th>Specific levansucrase activity† (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB 101 (lac+)</td>
<td>pHIPMalprom-lsc1</td>
<td>lsc1</td>
<td>56 ± 12</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom-lsc2</td>
<td>lsc2</td>
<td>21 ± 4</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom-lsc3</td>
<td>lsc3</td>
<td>70 ± 25</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom</td>
<td>No (empty vector)</td>
<td>Below detection</td>
</tr>
<tr>
<td>RA11r</td>
<td>pHIPMalprom-lsc1</td>
<td>lsc1</td>
<td>44 ± 8</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom-lsc2</td>
<td>lsc2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom-lsc3</td>
<td>lsc3</td>
<td>46 ± 7</td>
</tr>
<tr>
<td></td>
<td>pHIPMalprom</td>
<td>No (empty vector)</td>
<td>Below detection</td>
</tr>
</tbody>
</table>

* E. coli strain RA11r is melibiase-negative.
† Described in Section 2.2.
‡ Levansucrase activity was measured according to the release of glucose from 300 mM sucrose in cell extracts of *E. coli* transformants grown in LB-kanamycin medium to late-exponential growth phase, average activity ± standard deviation for two to six transformants is presented.

![Fig. 2. Lsc3-producing *Escherichia coli* gains the ability to grow on sucrose. *E. coli* HB101 (lac+) harboring pHIPMalprom-lsc3 was grown in liquid M9 minimal medium supplemented with kanamycin, auxotrophic supplements and either glucose or sucrose. The reference clone (Ref) carried the empty vector pHIPMalprom.](image)

Fig. 3. Non-denaturating polyacrylamide gel electrophoresis of *Pseudomonas syringae* pv. *tomato* DC3000 levansucrase proteins. Cell extracts (~15 µg protein per lane) of recombinant *E. coli* HB101 (lac+) (A and B) or RA11r (C) expressing individual levansucrases were separated in 7.5% polyacrylamide gel and stained with Coomassie brilliant blue (A) or soaked in McIlvaine’s buffer (pH 6.0) containing 0.015% Na-azide and 10% sucrose (B) or 10% raffinose (C). Only region with levan-synthesizing bands of gels B and C is shown. Clones harboring the empty vector were used as a reference (Ref).
and insoluble protein fractions of lsc3-expressing E. coli was run. Expectedly, levansucrase band was present only in the fraction of soluble proteins (data not shown). As discussed in [4], one way to avoid inactivation of the produced protein is using a promoter with appropriate strength. We conclude that PMAL1 has appropriate strength to produce an adequate amount of soluble catalytically active levansucrase protein in E. coli.

3.5. Cellular location of levansucrase in E. coli

Bacterial levansucrases are extracellular proteins in their native hosts [16,44]. Among levansucrases of Gram-negative bacteria, secretion mechanism is known only for LsdA of G. diazotrophicus. A 30-aa N-terminal signal peptide is cleaved off from LsdA during the transport to the periplasm, where the enzyme adapts its conformation, and thereafter it is transferred across the outer membrane without further proteolytic processing [44].

We analyzed the sequences of Lsc proteins of P. syringae DC3000 for the presence of a potential N-terminal signal peptide using a Signal P program version 3.0 [34]. No secretion leader was predicted for Lsc1, Lsc2 and Lsc3 proteins in contrast to LsdA of G. diazotrophicus that was analyzed as a reference. According to Li and Ulrich [16] computer analysis of levansucrases of P. syringae pv. glycinea PG4180 also predicted lack of N-terminal signal peptide [16]. In addition, the authors proved that levansucrases of PG4180 are not proteolytically processed during the secretion: the N-terminal amino acid sequence of levansucrase isoenzyme mixture from the supernatant of PG4180 was STSSAVSQLKNSPLAG-NINY, identical to the N-terminal sequence deduced from the nucleotide sequences of lscB and lscC of PG4180 (shown also in Fig. 1). The authors conclude that export of the levansucrases might therefore occur via a sec-independent mechanism. So, the function of N-terminal extension of 431-aa Lsc proteins (Fig. 1) has yet to be elucidated.

As growth media of lsc-expressing E. coli exhibited no levansucrase activity, distribution of one of the expressed levansucrases, Lsc3, between periplasmic and cytosolic fractions of E. coli was studied. It turned out that most (88%) of levansucrase activity was located in the cytoplasm. Compartment-specific reference proteins segregated correctly: (88%) of levansucrase activity was located in the cytoplasm. Fractions of levansucrases, Lsc3, between periplasmic and cytosolic levansucrase activity, distribution of one of the expressed processing [44].

across the outer membrane without further proteolytic enzyme adapts its conformation, and thereafter it is transferred off from LsdA during the transport to the periplasm, where the bacteria, secretion mechanism is known only for LsdA of G. diazotrophicus pv. glycinea PG4180 (shown also in Fig. 1). The authors conclude that export of the levansucrases of PG4180 is STSSAVSQLKNSPLAG-NINY, identical to the N-terminal sequence deduced from the nucleotide sequences of lscB and lscC of PG4180 (shown also in Fig. 1). The authors conclude that export of the levansucrases might therefore occur via a sec-independent mechanism. So, the function of N-terminal extension of 431-aa Lsc proteins (Fig. 1) has yet to be elucidated.

As growth media of lsc-expressing E. coli exhibited no levansucrase activity, distribution of one of the expressed levansucrases, Lsc3, between periplasmic and cytosolic fractions of E. coli was studied. It turned out that most (88%) of levansucrase activity was located in the cytoplasm. Compartment-specific reference proteins segregated correctly: majority of β-lactamase activity was detected in the periplasm and that of glucose-6-phosphate dehydrogenase in the cytoplasm (Table 3), proving reliability of cell fractionation data. We assume that a low amount of levansucrase in the periplasmic fraction of recombinant E. coli protects bacteria from toxic effect of sucrose. Indeed, according to our data, lsc-expressing E. coli exhibited undisturbed growth on sucrose (Fig. 2). As concluded from the analysis of the literature data, levan synthesis in a sucrose-growing Lsc-expressing E. coli can take place only in the periplasm. Namely, sucrose diffuses into the periplasm of E. coli through aspecific porins in the outer membrane, but cannot penetrate further being therefore absent from the cytoplasm [4,45]. Thus, cytosolic population of levansucrase will not get into contact with sucrose and thereby will not contribute to levan synthesis by the cells. This hypothesis agrees with severe toxicity of the expression of B. subtilis levansucrase (SacB) to E. coli growing in sucrose medium. SacB has mostly periplasmic location in E. coli [46] that should cause cell bursting due to synthesis of a high amount of levan in the periplasm if sucrose is present in the growth medium. In accordance with that, expression of Z. mobilis levansucrase caused no sucrose-toxicity to E. coli, because majority (73%) of the levansucrase was detected in the cytoplasm [47]. We hypothesize that in case of moderate levan synthesis in the periplasm, the polymer will somehow be excreted to growth medium, probably through pores in the outer membrane. We also suggest that in case of levan-synthesizing E. coli colonies, some levan on the colonies is probably produced by levansucrase released from dead cells.

3.6. All three levansucrases of P. syringae pv. tomato DC3000 use raffinose

In addition to sucrose, levansucrases of many bacteria, e.g. Z. mobilis [14,47–49], L. sanfranciscensis [40], L. reuteri [39] B. subtilis [50] and Microbacterium laevaniformans [11] can use a trisaccharide, raffinose. Raffinose utilization by levansucrases of P. syringae DC3000 was first suspected from colony phenotype of lsc-expressing E. coli RA11r on LB-raffinose plates. Regular strains of E. coli possess melibiase (α-galactosidase), splitting raffinose to galactose and sucrose, whereas the RA11r strain is melibiase-negative [20,21] having suitable background for levansucrase-mediated raffinose utilization assay. The lsc-expressing RA11r colonies turned mucoid during incubation on LB-kanamycin plates containing either sucrose or raffinose, the slime production being somewhat less pronounced in case of raffinose. Cell extracts of lsc-expressing RA11r produced reducing sugars from both, sucrose and raffinose, albeit with different relative activity: hydrolysis of 100 mM raffinose was 41–48% from the hydrolysis of 100 mM sucrose. The levansucrase activity assay on native polyacrylamide gels (Fig. 3) showed levan production from both, sucrose and raffinose.

One of the levansucrases, the Lsc3, was purified from recombinant E. coli HB101 (lac+) (see Section 2.8) for kinetic study of sucrose and raffinose utilization. The purification increased specific levansucrase activity on sucrose by more
did not stain. The chromatogram shown in Fig. 5 displayed and melibiose formed from sucrose and raffinose, respectively, phosphoric acid visualization method was used, thus glucose products were resolved on a silica gel. A fructose-specific urea–100 mM) of sucrose and raffinose for 6 min, and the reaction was incubated with four different concentrations (10, 20, 50 and 100 mM) of sucrose and raffinose for 6 min, the reaction was stopped and 1 µl aliquotes of reaction mixtures were chromatographed on a silica gel plate. 0.5 µl of 0.1 M solutions of standard sugars (fructose, sucrose, raffinose) were analyzed alongside. Fructose-containing sugars were visualized using urea–phosphoric acid treatment. F, fructose; S, sucrose; R, raffinose.

Fig. 4. Kinetics of sucrose hydrolysis by purified Lsc3 protein of P. syringae pv. tomato DC3000. Release of glucose from 5 to 300 mM sucrose was assayed in McIlvaine’s buffer (pH 6.0) with different concentrations of sucrose and raffinose for 6 min, the reaction was stopped and 1 µl aliquots of reaction mixtures were chromatographed on a silica gel plate. 0.5 µl of 0.1 M solutions of standard sugars (fructose, sucrose, raffinose) were analyzed alongside. Fructose-containing sugars were visualized using urea–phosphoric acid treatment. F, fructose; S, sucrose; R, raffinose.

than four-fold, up to the $V_{\text{max}}$ value of 286.2 ± 7.4 U/mg. This final activity value agrees with the predicted Lsc3 expression level in E. coli of about 20% from total soluble proteins (see Section 3.4). The $K_m$ for sucrose hydrolysis was 20.6 ± 2.1 mM and raffinose inhibited sucrose hydrolysis competitively (Fig. 4) with $K_i$ of 47.6 ± 5.0 mM. Thus, sucrose and raffinose probably share a binding site on the levansucrase. The $K_m$ for raffinose hydrolysis measured according to the liberation of reducing sugars was 41.3 ± 5.6 mM. Release of reducing sugars from 100 mM raffinose was 48% of the value obtained with 100 mM sucrose.

Next, a thin layer chromatography assay of levansucrase reaction products was carried out. The purified Lsc3 protein was incubated with four different concentrations (10, 20, 50 and 100 mM) of sucrose and raffinose for 6 min, and the reaction products were resolved on a silica gel. A fructose-specific urea–phosphoric acid visualization method was used, thus glucose and melibiose formed from sucrose and raffinose, respectively, did not stain. The chromatogram shown in Fig. 5 displayed different affinity of Lsc3 protein for sucrose and raffinose hydrolysis: in case of reaction with 10 mM sucrose, a clearly detected fructose spot is visible in a TLC plate, whereas reaction with 10 mM raffinose produces no detectable amount of fructose. Fructose liberation from raffinose was detected only at higher raffinose concentrations. TLC analysis also detects levan formation by Lsc3 at higher concentrations of sucrose, while levan production from raffinose was not observed at any raffinose concentration used in the assay (Fig. 5). Nevertheless, chromatography of samples from prolonged incubation (15 and 30 min) of Lsc3 with raffinose, indicated levan production at elevated raffinose concentrations, 50 mM and higher (data not shown). Literature data also show that levan synthesis by levansucrases is stimulated at high substrate concentrations [18,40]. Consistent with TLC analysis data, levan-producing activity of Lsc3 with sucrose is higher than with raffinose. The TLC method used in our assay can also resolve fructo-oligosaccharides [30]. As we were not able to detect a fructose-containing spot with mobility of fructo-oligosaccharides, they were probably not produced in a detectable amount from neither sucrose nor raffinose by Lsc3 under applied reaction conditions.

Levansucrase of Z. mobilis has been studied from the aspect of sucrose and raffinose utilization. The Z. mobilis enzyme has similar affinities for sucrose and raffinose [48,49], with capacity of raffinose hydrolysis exceeding that of sucrose hydrolysis [14,48] or being equal to that [49]. Unlike the Z. mobilis enzyme, levansucrases of P. syringae DC3000 prefer sucrose over raffinose. Interestingly, the only levansucrase protein purified from a P. syringae species, from pv. phaseolicola [18] is considered exceptional among bacterial levansucrase for its poor affinity ($K_m = 160$ mM) for sucrose hydrolysis. In contrast to that, the Lsc3 protein of P. syringae DC3000 described by us, had much higher affinity ($K_m = 21$ mM) for sucrose hydrolysis, being similar to respective value of levansucrases from many other bacteria, e.g. Bacillus sp. TH4-2 ($K_m = 16.7$ mM) [51], L. sanfranciscensis ($K_m = 13.1$ mM) [40], G. diazotrophicus ($K_m = 11.8$ mM) [8], L. mesenteroides ($K_m = 26.6$ mM) [12] and Erwinia herbicola ($K_m = 28$ mM) [52].

4. Conclusions

P_{MAL1}, the promoter of the H. polymorpha maltase gene possessing sigma 70-like sequence elements was demonstrated as suitable for the expression of biotechnologically relevant proteins in E. coli. As an example, three levansucrase proteins of P. syringae DC3000 were successfully produced in E. coli, and one of the recombinant proteins, Lsc3, was purified. Due to appropriate strength of the promoter, the Lsc1, Lsc2 and Lsc3 were produced in E. coli as soluble proteins in a high amount,
and formation of inclusion bodies was not observed. Kinetic assay of Lsc3 showed that a very low affinity for sucrose hydrolysis described for a levansucrase of *P. syringae* pv. phaseolicola [18] is not a common feature for levansucrases of many other bacteria. We also demonstrated that unlike the *P. syringae* pv. phaseolicola levansucrase [18], *P. syringae* DC3000 Lsc proteins could split and polymerize raffinose. The expression system worked out by us can be used to produce levansucrase proteins for structure–function analysis of wild-type and engineered levansucrases for both, basic research and practical applications. P*MAL1*, applied in this study, has additional attractive feature—besides *E. coli*, it is functional also in yeasts, e.g. *H. polymorpha* and *Saccharomyces cerevisiae* [1]. Thus, it can be used for the design of a common expression plasmids for more than just one host species, if plasmid replication and maintenance will be guaranteed. In this case, production of a protein of interest can be tested in different hosts to select the most appropriate one.

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