NOTES

Site-Directed Mutagenesis of Loop L3 of Sucrose Porin ScrY Leads to Changes in Substrate Selectivity

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The difference in substrate selectivity of the maltodextrin (LamB) and sucrose (ScrY) porins is attributed mainly to differences in loop L3, which is supposed to constrict the lumen of the pores. We show that even a single mutation (D201Y) in loop L3 leads to a narrowing of the substrate range of ScrY to that resembling LamB. In addition, we removed the putative N-terminal coiled-coil structure of ScrY and studied the effect of this deletion on sucrose transport.

The maltodextrin (LamB) and sucrose (ScrY) porins in the outer membranes of enteric bacteria allow efficient uptake of oligosaccharides even at low substrate concentrations (for recent reviews see references 14, 24, and 28). According to X-ray studies, both functional LamB (4, 13, 16) and ScrY (7) are trimers in which each monomer forms an 18-stranded antiparallel β-barrel. The β-sheets are connected by short turns toward the periplasmic side and by longer loops facing the exterior of the cell, with the exception of loop L3. This loop is folded into the lumen of the barrel and constitutes the central part of a constriction zone within the channel, which is directly involved in substrate recognition and binding. LamB seems to be optimized for maltose and maltooligosaccharides (1, 15, 26), whereas ScrY shows a broader substrate range which also includes sucrose (19, 22, 26). From crystallographic studies (7, 27) this difference in substrate selectivity is explained mainly by the wider pore of ScrY. In LamB, residues R109 and Y118 of loop L3 seem to sterically hinder the bulky sucrose molecule to permeate through the constriction zone. In ScrY these amino acids are replaced by the shorter residues N192 and D201, respectively (Fig. 1). In addition, D121 of LamB, which is thought to be involved in substrate binding (27), is also changed in ScrY (to F204), whereas the remaining residues lining the constriction zones of both porins are highly conserved. To study the contribution of these amino acids to the substrate selectivity of these porins we replaced them in ScrY and thus removes most of the amino-terminal extension of ScrY, including the putative coiled-coil structure. The replacement of the original 0.8-kb EcoRI-KpnI DNA fragment of pPSO112 (19), coding only for the amino-terminal part of ScrY, into pSU19 (12), yielding pUSL119 (Fig. 2). In detail, this fragment ranges from nucleotides −71 to +745, i.e., it carries the promoter scrYp and the first 229 amino acids of the ScrY precursor. Subsequently, a PCR was carried out with the divergent mutagenesis primer pair 8/27-3 and 8/27-5 (Fig. 2), leading to an amplified DNA fragment with two newly introduced BssHII restriction sites near each end. Cutting with BssHII and subsequent religation generated an in-frame deletion of 183 bp in scrY that did not cause any amino acid exchanges, as confirmed by sequencing. The deletion comprises base pairs 67 to 249 of scrY, i.e., amino acid residues 1 to 61 of the mature protein, and thus removes most of the amino-terminal extension of ScrY, including the putative coiled-coil structure. The replacement of the original 0.8-kb EcoRI-KpnI fragment of pPSO112 (ScrY−) with the deletion fragment resulted in pUSL112 (ScrYΔ61), which allows the adjustable expression of the mutant porin from the two regulated promoters scrYp and PscrYuno.

To generate the amino acid exchanges D201Y, F204D, and N192R, we cloned a subfragment containing the coding region of loop L3 from both pPSO112 (ScrY−) and pUSL112 (ScrYΔ61) into pAlter-1 (Promega). The mutagenesis procedure was carried out according to the supplier’s protocol with the mutagenesis primers 5′-CGAGGAAACACGGACATAAGA GTCAATCCA-3′ (D201Y), 5′-CCGGCCGGGTCCCAGCAC ATCAGAGTC-3′ (F204D), and 5′-GGTAACTCGAGATCT GTGCGGCTCGAAACG-3′ (N192R) and the N192R primer together with primer 5′-GGTACCGGGAGGTCCACGAC ATGAGGATCTC-3′ for the triple mutation (exchanged base pairs are underlined). The mutated region of scrY was
then transferred back into pPSO112 and pUSL112 to generate pPSO117 (ScrYD201Y), pPSO118 (ScrYF204D), pPSO119 (ScrYN192R), pUSL3113 (ScrY61-L3) and pUSL3213 (ScrYD61-L3) and sequenced as a control.

To check the expression levels of the different sucrose porin mutants we transferred the different scrY alleles into the LamB2 mutant PS9, a spontaneous λR Mal1 mutant from wild-type Escherichia coli K-12 (19). Outer membranes were isolated by sucrose gradient centrifugation, and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (19). Changes in the molecular weights of the deletion forms of ScrY could be observed (M_r about 45,000 compared to 53,000 for the wild-type porin), whereas the amounts of ScrY molecules in the

FIG. 1. Cross section through the constriction zone of LamB (left) and ScrY (right) viewed from the periplasmic side. The three amino acid residues of loop L3 that differ in both porins are marked. Data were derived from the Protein Data Bank, Brookhaven National Laboratory, and handled by using the program RasWin Molecular Graphics.

FIG. 2. Schematic representation of the construction of pUSL112 carrying the 5’ deleted scrY allele under the control of the hybrid promoter P_tac (3) and the scrY promoter P2. The deletion was introduced through two newly constructed BssHII restriction sites by PCR with the primer pair 8/27-3 (5’-GGTGCTTATATCCGTGGCGCGCATGGGCTGAGGCAGCGC-3’) and 8/27-5 (5’-GTGGCTCAGCGTACCGCGCCTTGAGAAAAAGCCG-3’) indicated by two divergent white arrows in the figure. The BssHII restriction sites in the primer sequences are underlined. The EcoRI-KpnI fragment from pPSO112 cloned into pUSL112 is marked by dashed lines. The promoters P_tac and P2 are not drawn to scale. B, BssHII; E, EcoRI; K, KpnI; S, SphI.
pressed sucrose-PTS (EIIScr), which was supplied by pPSO101 through the chromosomally encoded MalEFGK transport system. A single mutant (can be attributed to the D201Y exchange, as tested in the presence of the N-terminal deletion in ScrY this deletion lowered the transport rate increases, leading to a decrease of the \( K_m^{app} \) for the overall transport. The following results were obtained (also summarized in Table 1). (i) Neither the N-terminal deletion nor the amino acid changes in ScrY significantly changed the \( K_m^{app} \) for the maltose transport, indicating that the general function and particularly the permeability of maltose are not substantially impaired in the mutant sucrose porins. (ii) The triple mutation in L3 of ScrY increased the \( K_m^{app} \) of the sucrose transport about 25-fold to 100 \( \mu \)M, resembling the \( K_m^{app} \) of 85 \( \mu \)M for LamB. Most of this effect can be attributed to the D201Y exchange, as tested in the single mutant (\( K_m^{app} = 90 \mu \)M). The D201Y exchange obviously constricts the eyelet in a way similar to LamB, hindering the bulky sucrose molecules from passing through the outer membrane. The \( K_m^{app} \) for sucrose uptake from 100 \( \mu \)M (ScrY \( ^{L3} \)) to 30 \( \mu \)M (ScrY \( ^{61-L3} \)). Thus, in the absence of the coiled coil the diffusion of sucrose through the outer membrane seems to increase. At present we do not know whether this is due to an enhanced flux through the sucrose porin or whether the properties of the cell wall, including the general porins OmpF and OmpC, are influenced in some way.

It is conceivable that the amino-terminal extension of ScrY becomes more important under limiting conditions, e.g., during prolonged growth at low substrate concentrations or when the truncated porin is not overexpressed from a multicopy plasmid. We thus introduced the 5' deleted ScrY allele into an otherwise complete scr regulon which was then transposed onto a single-copy F plasmid. In particular, we started with plasmid pKJL710 (gift from K. Jähres, Osnabrück, Germany), which contains the scr regulon of pUR400 (18) cloned between two inverted repeats of the transposon Tn721 (21). The 1.0-kb SphI fragment of that plasmid carrying the N-terminal part of wild-type sucrose uptake of the corresponding truncated 0.8-kb SphI fragment of pUSL112 encoding ScrY \( ^{61} \). Both the mutated and the wild-type scr regulons were then allowed to transpose onto the F plasmid (6). The transposase of Tn721 was expressed from pPSO110, a derivative of pACYC184 (2) that carries the gene for the transposase (tnpA) under the control of the hybrid promoter Ptac. (3).

The F' plasmids containing either the wild-type (F'scr1) or the mutated (F'scr3) scr regulon were transferred by conjugation into E. coli K-12 strain S136 (20), and the presence of the different scrY alleles was confirmed by PCR. To test the influence of the N-terminal deletion in ScrY \( ^{61} \) on the function we measured the sucrose transport of induced and uninduced cells of both S136/F'scr1 and S136/F'scr3. As an internal control for appropriate induction levels of the scr regulons we also assayed the invertase activities of both strains (Table 2). No significant differences in sucrose transport and invertase activities were found between the two strains under these conditions, indicating again that the mutation in scrY has no or only a little effect on the function of the sucrose porin.

Growth competition experiments can reveal even minor changes in the fitness of bacteria exposed to selective environmental conditions. For example, low substrate concentrations strongly select for the optimization of transport activities (references in references 5, 8, and 10), and cells with even slightly improved porins should be enriched in such media. Therefore, we mixed equal amounts of strains S136/F'scr1 and S136/F'scr3 and incubated this mixture in 10 ml of a minimal medium containing either sucrose or glucose as the sole carbon source (5 \( \mu \)M). The cultures were inoculated with a titer of 10^3

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**TABLE 1. Apparent \( K_m \) values for sucrose and maltose uptake in strain PS9/pPSO101 (scrA') carrying different scrY alleles or lamB**, respectively*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Glycoporin</th>
<th>( K_m^{app} ) (( \mu )M) Sucrose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>250</td>
<td>400</td>
</tr>
<tr>
<td>pPSO112</td>
<td>ScrY</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>pTROY9</td>
<td>LamB</td>
<td>85</td>
<td>3</td>
</tr>
<tr>
<td>pPSO117</td>
<td>ScrYD201Y</td>
<td>90</td>
<td>9</td>
</tr>
<tr>
<td>pPSO118</td>
<td>ScrYF204D</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>pPSO119</td>
<td>ScrYN192R</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>pUSL3113</td>
<td>ScrY(^{L3})</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>pUSL312</td>
<td>ScrY(^{ΔL3})</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pUSL3213</td>
<td>ScrY(^{ΔL3})</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>

* Strain PS9/pPSO101 lacking LamB but expressing EIIScr constitutively from pPSO101 was used as the host for the different porin-carrying plasmids. For transport experiments cells were grown in Lennox broth (11) without glucose, containing the appropriate antibiotics for maintenance of the various plasmids as well as maltose (0.2% [wt/vol]) for the induction of the chromosomally encoded MalEFGK transport system. For induction of the sucrose porins, 2 mM concentrations of IPTG (isopropyl-\( β \)-D-thiogalactopyranoside) were added 1 h before cells were harvested at approximately \( 4 \times 10^8 \) cells per ml. Uptake was measured as described previously (17), with [\( ^{13} \)C]sucrose and [\( ^{13} \)C]maltose. \( K_m^{app} \) values were based on the initial uptake rates.

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**TABLE 2. Sucrose transport and invertase activity in strain S136 carrying either the wild-type (F'scr1) or the mutated (F'scr3) scr regulon on single-copy F8 plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Glycoporin</th>
<th>Induction*</th>
<th>Sucrose transport b</th>
<th>Invertase activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td>F'scr1</td>
<td>ScrY (^{+})</td>
<td>Noninduced</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>F'scr3</td>
<td>ScrY(^{ΔL})</td>
<td>Noninduced</td>
<td>25</td>
<td>4</td>
</tr>
</tbody>
</table>

* Cells were grown in minimal medium (25) with either glycerol (noninduced) or sucrose (induced for the scr genes) as the carbon source (0.2% [wt/vol]).

a Sucrose uptake and invertase activity were tested as described previously (17). Transport activities for sucrose (0.8 \( \mu \)M) are given in picomoles per minute per milligram of protein. Invertase activities are expressed in nanomoles per minute per milligram of protein.
cells per ml, grown for approximately 10 generations to stationary phase, diluted in fresh media to 10^7 cells per ml, and grown once more for a further 10 generations. We subsequently estimated the cell number ratios of both strains by PCR amplifying a DNA fragment that differed in length for products of 0.6 kb were expected with plasmid F' as a template carrying the wild-type scrY allele, and products of 0.6 kb were expected with plasmid F' scr3 containing the deleted form of scrY. Lanes 1 through 3, samples from a reconstruction experiment with premixed cultures of S136/F' scr1 and S136/F' scr3 without competitive growth. Strains were mixed to the following ratios of S136/F' scr1 to S136/F' scr3: 10:1 (lane 1), 1:1 (lane 2), and 1:10 (lane 3). Lanes 4 and 5, mixed cultures grown for about 20 generations in minimal media containing either glucose (lane 4) or sucrose (lane 5) as the sole carbon source (5 μM concentrations of each). The lengths of the fragments are indicated at the right.

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REFERENCES