

# Construction and in vivo analysis of new split lactose permeases

Wolfgang Wrubel<sup>a</sup>, Ursula Stochaj<sup>b</sup>, Ruth Ehring<sup>a,\*</sup>

<sup>a</sup>Institut fuer Genetik der Universitaet zu Koeln, D-50931 Koeln, Germany

<sup>b</sup>McGill University, Montreal, Que. H3G 1Y6, Canada

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## Abstract

The capacity of incomplete segments of *Escherichia coli* lactose permease to form transport-competent complexes in vivo was further tested. Two series of mutant *lacY* genes were constructed. One encoded N-terminal lactose permease segments of different length. The proteins specified by the other group contained deletions of different length and location within the N-terminal region. Several pairs of such mutant proteins reconstituted active lactose transport. For certain combinations duplications of protein segments were compatible with the formation of an active carrier. Duplication of helices could also be tolerated, when part of a single polypeptide chain.

**Key words:** LacY permease; Lactose transport; Functional complementation; Protein helix duplication

## 1. Introduction

The *Escherichia coli* lactose carrier represents a polytopic membrane protein that mediates proton-galactoside symport across the cytoplasmic membrane. Substrate binding, transport kinetics and amino acid residues important for transport activity have been studied in detail. Previous experiments support the idea that lactose permease is organized into 12 transmembrane  $\alpha$ -helices which are connected by hydrophilic mostly short loops ([1], and reviewed in [2,3]). Both the N-terminus and the C-terminus are exposed to the cytoplasmic face of the membrane [4,5]. Lactose permease is lacking a cleavable signal sequence, and we have shown that the protein becomes membrane-associated, before translation of the polypeptide chain is complete [6]. To identify regions important for the interaction with the lipid bilayer, we have previously generated plasmids encoding truncated forms of the protein or proteins carrying deletions within the N-terminal portion. Such mutant proteins are capable of membrane association independent of the missing part of the polypeptide [7].

The most rigorous proof for successful reconstitution of a split protein is demonstration of its biological activity. The analysis of functional complementation of membrane proteins was pioneered by Khorana's group with proteolytic fragments of bacteriorhodopsin [8]. We and others have previously shown that two incomplete fragments of lactose permease can insert into the cytoplasmic

membrane and gain a functional conformation that allows active transport of lactose [9,10].

Experiments in this study were designed to further analyze the capacity of N-terminal fragments of lactose permease to interact with different incomplete fragments of the lactose carrier. Towards this end, we generated various combinations of incomplete LacY polypeptides that have not been analyzed so far. Our results describe new split permeases that were competent for membrane association and the assembly of an active carrier. We have identified sequence duplications so far not described in other studies [11] that are tolerated for the reconstitution of transport-competent lactose carriers.

## 2. Materials and methods

### 2.1. Radioactive materials and enzymes

These compounds were purchased from sources indicated previously [9]. Enzymes were used as recommended by the suppliers.

### 2.2. Bacteria and plasmids

Strains T184, DS410 and plasmid pVI-I carrying the wild-type *lacY* gene have been described [7]. The transport-negative *E. coli* strain T184 served as the host for plasmid constructions, preparation of plasmid DNA and for transport assays. The RecA-phenotype of T184 was verified by analysis of its UV sensitivity. Minicells were prepared from *E. coli* strain DS410 [12]. Plasmid pVI-1 carries the *lacY* insert of plasmid pGM21 [13], the original source for all mutant *lacY* genes used in this study. Derivatives with unique restriction sites for *Bss*HII following codon 143 and for *Sma*I following codon three, respectively, have been described elsewhere [7,9]. To generate the vector plasmid pV142, non-essential sequences were deleted from pACYC184 [7]. All *lacY* alleles were under control of the authentic *lacOP* region and cells carried the *lacI*<sup>s</sup> allele on an F'-episome to ensure overexpression of the Lac repressor. The translation started at the original initiation site and each mutant protein contained at least the three N-terminal amino acid residues of authentic lactose permease.

### 2.3. Construction of plasmids

For simplicity plasmids are referred to by the *lacY* gene they encode [7]: truncated polypeptides are identified by the last codon of the au-

\*Corresponding author. Fax: (49) (221) 41-3292.

**Abbreviations:** CCCP, carbonylcyanide *m*-chlorophenylhydrazide; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; NEM, *N*-ethylmaleimide; TDG, D-galactopyranosyl- $\beta$ -D-thiogalactopyranoside.

thentic *lacY* sequence [14] they contain. The names for proteins with deletions indicate the first and the last codon that has been deleted.

Genes for N-terminal truncated segments of lactose permease were constructed from plasmid pY174 [7] which encodes amino acid residues 1 to 174 of the carrier. Plasmid pY174 was linearized with *Ban*II and incubated with exonuclease *Bal*31 for various times. Subsequently, *Nhe*I-linkers (12mer, New England Biolabs) carrying amber codons in all six reading frames were inserted (Fig. 1). To delete non-essential pBR322-sequences, DNA was digested with *Sty*I and *Nhe*I followed by religation with an appropriate synthetic DNA-adapter. The generation of mutant *lacY* genes encoding truncated proteins Y50, Y71/1 and Y143 has been described previously [7]. For combination of these proteins with polypeptides carrying internal deletions, the *Eco*RI-*Bgl*II-fragments of the respective genes were cloned into the *Eco*RI-*Sty*I sites of pBR322. A different control plasmid, pBR322dtet, carried a deletion of the tetracycline resistance gene. This plasmid was obtained by digestion of pBR322 with *Eco*RI and *Sty*I followed by filling in the recessed ends and autoligation.

The deletion mutants  $\Delta Y72$ –142 [7] and  $\Delta Y4$ –69 [9] have been described previously. To generate mutants  $\Delta Y4$ –45 and  $\Delta Y4$ –98, *Sma*I restriction sites were introduced at codons 45 or 98 of the *lacY* sequence by polymerase chain reaction (PCR) mutagenesis. These sites in conjunction with the *Sma*I site at codon three [9] permitted deletion of the sequences extending from codon 4 to 45 and from codon 4 to 98, respectively.

A series of internal *lacY* deletions of different length starting at position 72 and extending up to position 142 was obtained by linearizing plasmid pVI-1 at the unique *Ava*I site at position 71 of the *lacY* sequence followed by *Bal*31 digestion and insertion of *Sma*I linkers. The position of the linkers was determined by DNA sequence analysis. *Sma*I-*Bss*HII fragments of suitable length were used to replace the *Ava*I-*Bss*HII segment of the wild-type *lacY* gene. Restoration of the correct reading frame was achieved by digestion of protruding *Ava*I ends or by filling in recessed ends, as required. All deletion mutants are derivatives of plasmid pACYC184.

The linkers used for construction of various plasmids introduced additional amino acid residues that are not part of the wild-type sequence. For mutant Y106/1, a serine followed residue 106 of the lactose carrier; protein Y141/2 carried an additional proline and serine residue. After residue three the sequence Phe-Pro-Arg-Ala is inserted in  $\Delta Y4$ –45 and Phe-Pro-Arg-Val in  $\Delta Y4$ –98, and  $\Delta Y72$ –120 contains an additional arginine residue between positions 71 and 121. The sequence of proteins Y71/1 and  $\Delta Y4$ –69 has been described [9]. In addition to co-existing plasmids, 'combination plasmids' carrying two mutant *lacY* genes on a single plasmid were generated for the combination 1,4 and 6 in Fig. 2 essentially as described previously [9]. All combination plasmids were derived from plasmid pACYC184.

To construct a sequence duplication within a single polypeptide chain, a *Sma*I-*Bss*HII fragment was isolated from a mutant *lacY* gene carrying a *Sma*I site following codon three [9]. This fragment was cloned into the *Ava*I-*Bss*HII sites of the wild-type gene. The resulting plasmid pY121234 encoded a carrier with duplicated  $\alpha$ -helices I and II followed by the authentic lactose permease sequence. Plasmid pY1212 was obtained by deletion of sequences encoding helix III and IV from pY121234. (The different mutant lactose carriers are depicted in Fig. 3.) Both mutant plasmids are derivatives of pACYC184. Their names represent the helix composition of the N-terminal region with respect to the wild-type protein Y1234. For both proteins the newly created loop between the first helix II and the second helix I contains glycine in position 71 followed by an additional glycine, which is not part of the authentic sequence, and lysine 5 preceding the second helix I. The region containing the second helix II in Y1212 ends with amino acid residue 71 and is directly joined to the C-terminal portion of lactose permease starting at residue 127 of the authentic sequence (see Fig. 1 and Fig. 3).

The correct DNA-sequence of newly established junctions for mutant *lacY* genes was verified by sequencing with the sequenase 2.0 kit (USB, Bad Homburg v.d.H.).

#### 2.4. Transport assay

Uptake of [ $^{14}$ C]lactose was determined essentially as described previously [9]. For each test the cells were incubated for 3 min with [ $^{14}$ C]lactose (0.1 mM final conc.). Radioactivity taken up during this period was determined by liquid scintillation counting.

Deviations from the procedure published previously were as follows: (i) the concentration of inducer (IPTG) was raised to 2.0 mM; (ii) washed cells were resuspended in transport buffer to an optical density of 3.5 (600 nm, 1 cm light path); (iii) to determine the uptake of radiolabeled lactose, cells were collected on glassfiber filters (GF/F; Whatman Scientific Ltd., Maidstone, UK [15]).

Under these conditions cells carrying the *lacY*-wild-type plasmid pVI-1 accumulated approximately 40 nmol lactose/mg cell protein. Values given in Figs. 2 and 3 represent measurements of several independent transformants and retransformants. For the inactive combinations  $\Delta Y4$ –45 + Y50 and  $\Delta Y72$ –120 + Y70-141/2 only primary transformants have been analyzed.

For all deletion proteins that were active when combined with truncated proteins (Fig. 2), we found that pairwise combinations with other truncated proteins allowed the formation of transport competent carriers although with lower activity (data not shown). Truncated proteins that restored transport were only slightly different in size as compared to those shown in Fig. 2. In contrast, several other truncated polypeptides reconstituted only marginal transport or did not restore carrier activity (not shown).

#### 2.5. Analysis of plasmid-encoded proteins

Most of the mutant *lacY* derivatives were studied in *E. coli* minicells. De novo synthesized proteins were biosynthetically labeled and separated by SDS polyacrylamide electrophoresis [16]. The mutants described in this study produced proteins of the expected electrophoretic mobility. In all cases the amount of mutant protein was lower than obtained for the wild-type carrier. This indicates that the relative transport activities given for the active mutants (see below) underestimate the specific transport activities of the LacY-related proteins. For deletion mutants lacking more than the first three helices, proteins could not be detected by this assay. These mutants were not further analyzed.

### 3. Results and discussion

#### 3.1. Screening for functional interaction between incomplete *LacY* segments

Simultaneously synthesized incomplete protein segments of lactose permease ('split permease') can reconstitute functional carriers that accumulate lactose in vivo [9,10]. In contrast, none of the individual mutant proteins was able to restore lactose accumulation. The combination of incomplete LacY proteins encoded by plasmid pAY (Fig. 2, line 1) served as a prototype for further studies presented here.

To extend our analysis we have now constructed two series of new mutant *lacY* genes. Series one encoded different N-terminal segments of lactose permease, referred to as truncated proteins. The second series coded for proteins harboring deletions of different length and location within the N-terminal region of the carrier. To screen for functional complementation, T184 cells were transformed with co-existing plasmids encoding different proteins of series one and two. To demonstrate that the carrier activity was plasmid-linked, we re-isolated plasmid DNA from primary transformants and retransformed T184. Retransformed cells and primary transformants exhibited comparable transport. For some of the split permeases we have generated combination plasmids (see section 2) that carry both mutant *lacY* alleles on the same plasmid. Similar results were obtained for transport with these combination plasmids.

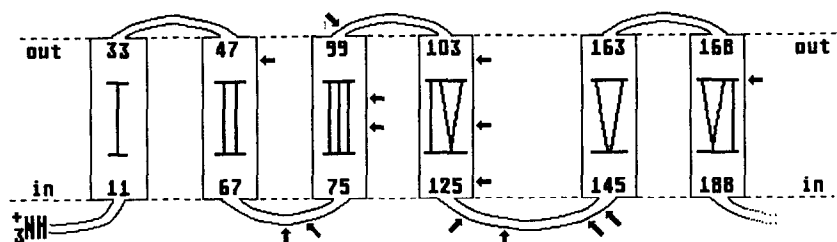


Fig. 1. Schematic representation of the N-terminal part of lactose permease. The rectangles symbolize the predicted  $\alpha$ -helices. Arabic numbers at the top and the bottom of each rectangle indicate the first and the last amino acid of each helix (taken from [3]). The arrows show the positions of the premature translation stop of the different truncated proteins.

### 3.2. Reconstitution of active carrier from two complementary segments

The two artificial subunits of lactose permease encoded by plasmid pAY contained almost no sequence duplication (Fig. 2, line 1). Similarly, a different combination, protein  $\Delta Y4-98$  and the truncated polypeptide Y106/1, showed an only slightly minor transport. Interestingly, both proteins  $\Delta Y4-98$  and Y106/1 are predicted to contain odd numbers of  $\alpha$ -helices. The interruption of the polypeptide chain is most likely at the periplasmic face of the membrane (Fig. 2, line 2). Since we obtained transport for this split carrier, it is reasonable to assume that its membrane topology resembles wild-type permease. Thus, the last helix of the truncated protein carrying the newly created C-terminus and the first helix of the deletion protein containing the new N-terminus are assumed to insert as individual helices into the lipid bilayer.

This type of membrane association has been suggested as the two-stage model [17], which proposes that membrane insertion of single hydrophobic helices is followed by the interaction between different parts of the protein within the lipid bilayer. Single hydrophobic helices of the carrier showed autonomous membrane association when part of LacY-PhoA fusion proteins [18]. For several *lacY*-deletion mutants with odd numbers of helices the C-terminus resides on the cytoplasmic face of the membrane as known for the wild-type protein [19]. This result may be explained by: (a) insertion of single helices into the lipid bilayer; or (b) topological 'frustration' as described recently for mutant forms of *E. coli* leader peptidase [20].

We also generated mutant carrier proteins for which the interruption of the polypeptide chain between helix one and two is most likely located at the periplasmic side of the membrane. Co-synthesizing the respective mutant proteins,  $\Delta Y4-45$  and Y50, did not restore transport under our conditions (Fig. 2, line 3). According to a preliminary report (McKenna, E., Bibi, E., Hardy, D., Pastore, J.C. and Kaback, H.R. (1992) Biophys. J. 61, A287 (abstr.)), a similar combination of LacY proteins restored active transport, when the incomplete proteins were overexpressed at a high rate.

### 3.3. Reconstitution of active split permeases containing sequence duplications

Co-synthesis of the deletion protein  $\Delta Y4-69$  and the truncated polypeptide Y143 which is predicted to comprise the first four  $\alpha$ -helices of lactose permease, failed to restore transport [9]. One possible explanation for this result was that the duplication of the very hydrophobic third and fourth  $\alpha$ -helix in this combination might perturb the functional interaction of protein segments.

To analyze the effect of such sequence duplications within the N-terminal region of split permeases, deletion proteins were co-synthesized with different truncated proteins. In each case, the truncated polypeptides contained the protein sequence lacking in the deletion partner and additional sequences of various lengths. These additional sequences were present in both the truncated and the deletion protein and are referred to as sequence duplications.

Simultaneous synthesis of  $\Delta Y4-69$  and the truncated protein Y106/1 (ending close to the start of helix four) reconstituted a transport-competent lactose carrier. The transport amounted to 5–10% of the wild-type level (Fig. 2, line 4). In this combination, helix three is predicted to be present in both proteins. Therefore, the transport-competent complex most likely contained 13 helices. In contrast to Y106/1, longer LacY proteins or truncated polypeptides ending within the third hydrophobic stretch showed no or only marginal complementing activity when co-synthesized with  $\Delta Y4-69$ .

The sequence duplication of the second  $\alpha$ -helix was also compatible with the formation of an active complex, i.e. protein  $\Delta Y4-45$  and the truncated polypeptide Y71/1 (Fig. 2, line 5) reconstituted transport activity similar to the combination of protein  $\Delta Y4-69$  with Y71/1. At present, however, we cannot exclude that the sequence duplications have been removed or shortened by partial proteolysis of the mutant polypeptide chain.

We generated another class of deletion mutants: in each case the deletion started after the second helix (codon 72) and removed at least helix three and the first half of helix four. Different isolates contained further extensions of the deletion up to the beginning of helix

five (see Fig. 1). The highest activity (5–10%) was observed for deletion protein  $\Delta Y72-120$  in combination with the truncated protein Y141/2 (Fig. 2, line 6). Lower transport was also obtained for several other combinations with truncated proteins that comprised the first four helices and the adjacent loop. In contrast, the combination of these deletion proteins with truncated polypeptides comprising only helix three and four and the adjacent loop did not result in the assembly of an active lactose carrier (exemplified by the combination shown in Fig. 2, line 7). Both proteins of the complementing pair  $\Delta Y72-120$  and Y141/2 contained the first two  $\alpha$ -helices of wild-type permease, indicating that a duplication of this segment still permitted a 'meaningful' assembly of a split permease.

Similar observations have been made for the func-

tional complementation of co-synthesized portions of the FhuB iron(III) hydroxamate transport protein from *E. coli* and with bacteriorhodopsin regenerated from proteolytic fragments. Sequence duplications of these membrane proteins did not prevent the reconstitution of activity [21,22]. In contrast to the pairs of split permeases presented in this study, transport-active combinations described by Bibi and Kaback [11] harbored extensive sequence duplications. The refore these complexes were predicted to resemble lactose dimers rather than truly split permeases.

### 3.4. Transport-competent lactose carrier containing a sequence duplication within a single polypeptide chain

The active carrier generated by the combination of  $\Delta Y72-120$  and Y141/2 is predicted to contain 14 helices.

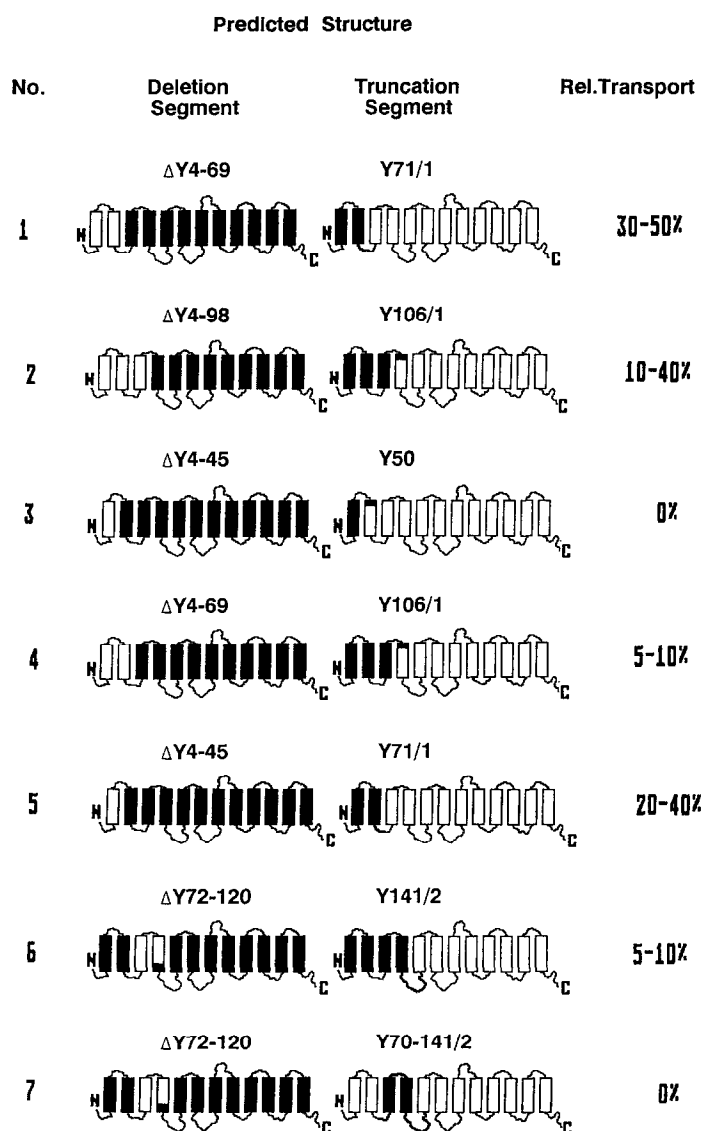


Fig. 2. Scheme of split permeases. The organization of mutant proteins was deduced from secondary structure models predicted for the wild-type protein. Parts of lactose carrier present in the mutant proteins are indicated by filled rectangles. They are drawn as predicted segments of the intact protein. Open rectangles represent deletions of the protein or portions that are missing due to premature termination of translation. Relative transport compared with cells harboring the *lacY* wild-type plasmid pVI-1 (see section 2).

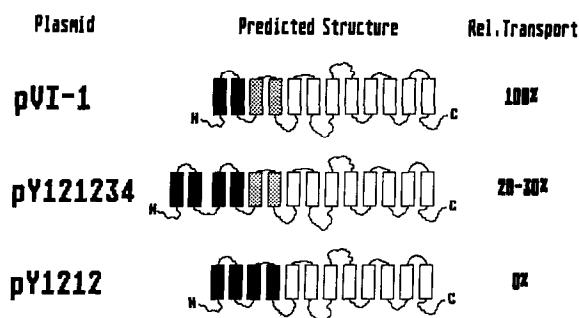


Fig. 3. Predicted structures and transport activities of the LacY proteins encoded by the plasmids pY121234 and pY1212 compared to the wild-type carrier (Y1234) encoded by plasmid pVI-1. Filled in rectangles represent  $\alpha$ -helices one and two, dotted rectangles the helices three and four, and helices five to twelve are shown as open rectangles. For measurements of the relative transport see section 2.

Another *lacY* derivative harboring a sequence duplication was the LacY mutant protein Y121234. It contained two copies of the helices one and two followed by the authentic sequence of lactose permease (Fig. 3, line 2). This mutant carrier most likely also consists of 14 helices. In contrast to the transport-restoring complex of  $\Delta Y72-120$  and Y141/1, it is organized into a single polypeptide chain. T184 cells synthesizing Y121234 regained about 20% to 30% of the wild-type activity. Transport measured for the mutant carrier showed characteristics similar to wild-type permease [23]. Dissipation of the transmembrane proton gradient by CCCP reduced drastically the active uptake of lactose. In addition, uptake of lactose was inhibited by the high affinity substrate TDG, and transport activity was lost upon treatment of cells with the SH-specific reagent NEM.

So far, however, it was not possible to replace helices three and four by the region comprising the first two helices. Mutant protein Y1212 was obtained by deleting the region of helices three and four of protein Y121234 leading to a permease most likely consisting of 12 helices, which contained a tandem repetition of the first two helices (Fig. 3, line 3). This protein did not restore any transport activity, although the synthesis of the polypeptide was detected in *E. coli* minicells.

### 3.5. Conclusions

Lactose permease belongs to the major facilitator superfamily which contains prokaryotic and eukaryotic transport proteins of common evolutionary origin [24]. It has served as a model to analyze transport mechanisms and to develop methods to study the structural organization and biosynthesis of membrane proteins. Our results support the idea that different regions of the lactose carrier can autonomously associate with the cytoplasmic membrane. Moreover, we have defined new combinations of split permeases that can functionally interact to yield active carriers. Models for the three-

dimensional organization of the protein will have to account for the high degree of flexibility exhibited by lactose permease. Active carrier can be assembled from different combinations of incomplete polypeptide chains, and various sequence duplications can be tolerated for split permeases and also within a single polypeptide chain.

The generation of split proteins has been successfully employed to study proteins of prokaryotic and eukaryotic origin. Split versions of different integral membrane proteins of pharmacological interest, such as the tetracycline resistance gene product [25], the  $\beta$ -adrenergic receptors [26], the muscarinic receptors [27] and a sodium channel [28] have been analyzed which demonstrates the general applicability of this approach.

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