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Ligand binding analyses of the putative peptide transporter YjdL from *E. coli* display a significant selectivity towards dipeptides

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ABSTRACT

Proton-dependent oligopeptide transporters (POTs) are secondary active transporters that couple the inwards translocation of di- and tripeptides to inwards proton translocation. *Escherichia coli* contains four genes encoding the putative POT proteins YhiP, YdgR, YjdL and YbgH. We have over-expressed the previously uncharacterized YjdL and investigated the peptide specificity by means of uptake inhibition. The IC_{50} value for the dipeptide Ala-Ala was measured to 22 mM while Ala-Ala-Ala was not able to inhibit uptake. In addition, IC_{50} values of 0.3 mM and 1.5 mM were observed for Ala-Lys and Tyr-Ala, respectively, while the alanyl-extended tripeptides Ala-Lys-Ala, Ala-Ala-Lys, Ala-Tyr-Ala and Tyr-Ala-Ala displayed values of 8, >50, 31 and 31 mM, respectively. These results clearly indicate that unlike most POT members characterized to date, including YdgR and YhiP, YjdL shows significantly higher specificity towards dipeptides.

Introduction

Peptide uptake in *Escherichia coli* is mediated by three genetically different types of transport systems: the dipeptide permeases (Dpp), the tripeptide permeases (Tpp) and the oligopeptide permease (Opp). Common for all three is that in general, members of these families are able to transport both di- and tripeptides. In the case of Opp however, up to hexapeptides are translocated. Dpp and Opp are multicomponent primary transporters, which require the hydrolysis of ATP for transport. The Tpp in contrast consists of a single polypeptide and belongs to the group of secondary active transporters. The Tpps have been conserved onto mammals, in contrast to the Dpps and Opps [1].

Secondary active transporters constitute one of the largest groups of transporters. These transporters are able to utilize a secondary source of energy, *i.e.* an electrochemical ion gradient to accumulate vital substances such as saccharides, amino acids, nucleobases, ions, peptides etc. Proton-dependent oligopeptide transporters (POTs, http://www.tcdb.org/) are secondary active symporters that typically facilitate the uptake of a range of different di- and tripeptides by coupling it to the simultaneous energetically favorable uptake of protons. The POTs are found in a wide range of organisms from bacteria to mammals. Based on sequence analyses, the POTs have been categorized as a Major Facilitator Superfamily (MFS)-like family [2]. The MFS include the biochemically and structurally well-characterized transporter lactose permease [3]. The topology of the POTs has been shown experimentally to consist of 12 transmembrane helices with inwards facing N- and C-termini [4,5].

E. coli contains four genes encoding the putative POT proteins YhiP, YdgR, YjdL and YbgH. They all appear to have relatively low amino acid sequence identity with their human homolog peptide transporter 1 (hPept1; 21–23%), the most studied POT member, and mutually cluster in the pairs YdgR–YhiP (51% identity) and YjdL–YbgH (56% identity) with approximately 26–28% identity between them. While YhiP and YdgR have both been experimentally verified to be POTs and have substrate specificities homologous to hPept1 [6,7], the YjdL and YbgH remain uncharacterized.

In the study presented here we have cloned, over-expressed and characterized the ligand preference of the putative POT YjdL from *E. coli*. Our results clearly indicate that YjdL has a significantly higher specificity towards dipeptides compared to tripeptides.

Materials and methods

Molecular biology. For construction of expression vector (pTTQ18-*yjdL*), the primers 5′CAGCAGAATTCGAAAACACCCTCACA GCCGCGCGCGATATACTATATCGTGGCGATCCA3′ and 5′ACCCAAGC TTTTAATGGTGATGGTGATGGTGATCGTTGCTCTCCTGTATCATATT3′

Abbreviations: POTs, proton-dependent oligopeptide transporters; MFS, major facilitator superfamily; Dpp, dipeptidepermeases; Tpp, tripeptidepermeases; Opp, oligopeptidepermease

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were used to amplify the ORF from the *E. coli* MG1655 genomic DNA using 'touchdown' PCR [8]. The EcoRI, and HindIII sites and oligonucleotides encoding hexa-Histag were introduced at the 5′ and 3′ ends of the *yjdL* gene, respectively. The PCR product, digested with EcoRI/HindIII, was then inserted into the corresponding sites of pTTQ18 [9] to generate a pTTQ18-*yjdL* construct. The construct was verified by DNA sequencing.

Expression. A single colony of *E. coli* BL21(DE3)pLysS cells harboring the plasmids pTTQ18-*yjdL* or pTTQ18 were inoculated in 5 mL LB-media containing 100 μg/mL Ampicillin and 40 μg/mL chloramphenicol and allowed to grow over night. Subsequently a 1:50 dilution of the overnight culture in 10 mL LB-media containing antibiotics as above was left to grow for approximately 3 h to reach an OD_{600} of 0.6–0.8 and hereafter induced by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested after an additional growth of 3 h.

Isolation of solubilized membranes. Cells from 1 L expression culture were re-suspended in lysis buffer (20 mM Tris–HCl pH 7.6, 300 mM NaCl, 5 mM Imidazole, 30 μg/mL DNAse and one Complete Protease Inhibitor Cocktail tablet/50 mL buffer (Roche)) and lyzed by two passes through a cell disruptor (Constant Systems Ltd.) operating at 33 kpsi. Cell debris was removed by centrifugation at 30,000*g* for 25 min and membranes were subsequently isolated by centrifugation at 100,000*g* for 60 min. Membrane pellets were re-suspended in lysis buffer containing 2.5% *n*-dodecyl-β-D-maltoside (Anatrace) and solubilized under stirring for 30 min. Finally, the solution was cleared for insoluble material by centrifugation at 100,000*g* for 30 min.

Western blot. Solubilized membrane fractions were separated by SDS–polyacrylamide electrophoresis on a 10% Bis–Tris gel and subsequently blotted onto a polyvinylidenedifluoride membrane using an XCell II blotting module (Invitrogen). Immunodetection of the His-tagged recombinant protein was performed using the Penta-His HRP Conjugate kit (Qiagen) followed by SuperSignal® West Pico chemiluminescent substrate (Pierce). Signals were detected using a FluorChem® HD2 imaging system (Alpha Innotech).

Uptake Assay. The assay was performed essentially as described by Weitz et al. [6] with adjustments of the amount of cells and concentration of the dipeptide derivative β-Ala-Lys-AMCA (Biotrend). Briefly, the harvested cells were re-suspended in modified Krebs-buffer (25 mM Hepes/Tris pH 7.4, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose) to an optical cell density (600 nm) of 5×10^9 cells/mL. The assay volume of 100 μL contained 10 μL of 0.1 mM fluorescent β-Ala-Lys-AMCA stock solution, 50 μL of various concentrations of the competitor/substrate solutions or modified Krebs-buffer, and 40 μL of bacteria cells. All ligands (purchased from Sigma–Aldrich, Bachem or custom synthesized from CASLO) dilutions were prepared with Krebs-buffer. All uptake experiments were performed at 37 °C for a period of 60 s. After incubation, 500 μL ice-cold Krebs-buffer was added to stop uptake. The cells were pelleted and washed twice with ice-cold modified Krebs-buffer and subsequently dissolved in 100 μL modified Krebs-buffer. Nonspecific uptake control experiments with empty pTTQ18 vector transformed *E. coli* cells were performed under the same conditions and procedures as described above. Uptake was quantified by fluorescence measurements (excitation at 340 nm and emission 460 nm) using a Safire 2 fluorimeter. Specific β-Ala-Lys-AMCA uptake was determined by deducting unspecific uptake from total uptake.

All measurements were done in triplicates and all experiments were reproduced at least three times independently. Data analyses were performed using Graphpad Prism. All assay-related figures are a representative of three similar experiments.

Results

Our objective in this study was to over-express and investigate the substrate specificity of YjdL from *E. coli*. The preliminary results from uptake alanyl-peptide inhibition studies indicated that YjdL displays a lower specificity towards Ala-Ala-Ala compared to Ala-Ala. The subsequent experiments were designed to investigate this, among POTs, unusual property further.

Expression

YjdL was over-expressed in *E. coli* using the pTTQ18 vector. This vector has been shown to be particularly effective for over-expression of bacterial secondary transporters [10]. The final protein product is expressed as a fusion with a C-terminal hexa-His tag, in light of topology informed strategies for expression of membrane proteins in *E. coli* [11]. This tag was included for detection by Western blotting. Immunostaining of solubilized membrane fractions using a penta-HRP conjugate clearly showed a band at 37 kDa, not present in the membrane fractions of cells harboring the empty vector, corresponding to the over-expressed protein (Fig. 1A). Anomalous migration of secondary transporters in SDS-gels (calculated Mw of YjdL 53.9 kDa) is a common phenomenon, possibly caused by their hydrophobicity, high binding of SDS or the retention of secondary/tertiary structure facilitating their passage through the gel [12].

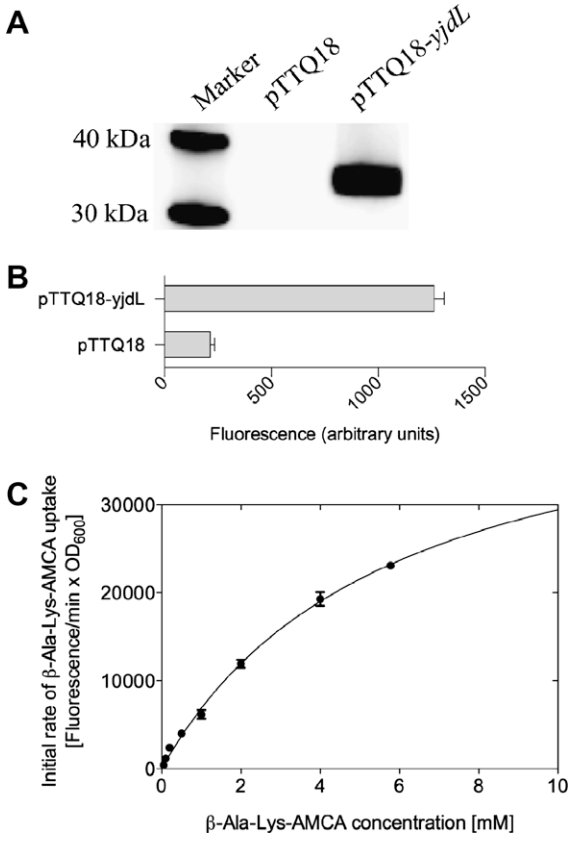

Fig. 1. (A) YjdL expression analyzed by Western blot showing the protein marker (MagickMark XP), solubilized membranes of IPTG induced *E. coli* BL21(DE3)pLysS cells harboring an empty pTTQ18 vector and the pTTQ18-*yjdL*, respectively. (B) β-Ala-Lys-AMCA uptake by IPTG induced *E. coli* BL21(DE3)pLysS cells harboring the plasmids pTTQ18 and pTTQ18-*yjdL*, respectively. The cells were incubated with modified Krebs-buffer containing 100 μM β-Ala-Lys-AMCA. (C) Saturation curve of β-Ala-Lys-AMCA specific uptake (normalized to number of cells) plotted as a function of substrate concentration. Uptake was measured at concentrations between 100 μM and 5.78 mM (maximal concentration achievable).