Biochimica et Biophysica Acta 1864 (2017) 393–398

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Critical role of the proton-dependent oligopeptide transporter (POT) in the cellular uptake of the peptidyl nucleoside antibiotic, blasticidin S

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ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form 22 November 2016
Accepted 29 November 2016
Available online 1 December 2016

Keywords:
POT family
Oligopeptide transporter
Blasticidin S
Peptidyl nucleoside
Ptr2
Yeast

ABSTRACT

Blasticidin S (BlaS) interferes in the cell growth of both eukaryotes and prokaryotes. Its mode of action as a protein synthesis inhibitor has been investigated extensively. However, the mechanism of BlaS transport into the target cells is not understood well. Here, we show that Ptr2, a member of the proton-dependent oligopeptide transporter (POT) family, is responsible for the uptake of BlaS in yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Notably, some mutants of Ptr2 that are dysfunctional in dipeptide uptake were still competent to transport BlaS. Mouse-derived oligopeptide transporter PepT1 conferred BlaS sensitivity in the *S. cerevisiae ptr2*Δ mutant. Furthermore, bacterial POT family proteins also potentiated the BlaS sensitivity of *E. coli*. The role of the POT family oligopeptide transporters in the uptake of BlaS is conserved across species from bacteria to mammals.

1. Introduction

Blasticidin S (BlaS) is an antibiotic produced by *Streptomyces griseochromogenes* [1]. BlaS was first used in agriculture as a natural non-mercurial fungicide to kill *Pyricularia oryzae*, the fungus that causes blast disease in rice. BlaS is a broad spectrum antibiotic and inhibits cell growth in prokaryotes, fungi, plants, and mammalian cells. BlaS occupies the P-site of the large ribosomal subunit and induces conformational changes in tRNA at the P site, thus effectively inhibiting protein synthesis [2]. Structurally, BlaS is a peptidyl nucleoside in which cytosine and modified arginine are linked to deoxyglucuronic acid [3]. Since this cytidine moiety binds to the corresponding guanine base at the P-site, cytosine is crucial for the inhibitory action of BlaS. Some microorganisms, which are naturally resistant to BlaS, produce an enzyme that converts BlaS to an inactive form by deaminating the pivotal cytosine moiety. Two non-homologous genes encoding the BlaS deaminase, named *bsd* and *bsr*, were isolated from *Aspergillus terreus* and *Bacillus cereus*, respectively. Heterologous expression of these BlaS deaminases confers resistance on BlaS-sensitive host cells. This trait enables the utilization of the *bsd* and *bsr* genes as dominant selectable markers. Combination of BlaS and the genes that confer BlaS resistance is now widely used for gene transfer [4–7].

Although the molecular mechanism of how BlaS inhibits cell growth has been investigated extensively, molecular mechanisms involved in the cellular uptake or transport of BlaS are still not fully understood. Recently, it has been reported that inactivation of a leucine-rich repeat-containing protein 8D (LRRC8D) confers resistance to BlaS in cultured mammalian cells [8]. The membrane protein LRRC8D is one of the subunits of a heteromeric complex called volume-regulated anion channel (VRAC) that regulates cell volume in response to changes in the concentration of external osmolytes [9]. However, LRRC8 protein is found only in chordates, and not evolutionarily conserved in bacteria, plants, and lower eukaryotes such as fungi, in spite of the fact that BlaS actively kills all these organisms. Therefore, non-chordates might be expressing a different BlaS transporter.

The proton-dependent oligopeptide transport (POT) family proteins, also termed the peptide transporter (PTR) family, are conserved in all organisms except the Archaea [10–13]. POT family proteins, including mammalian PepT1 and PepT2, are responsible for the uptake of extracellular di- and tripeptides, a variety of peptidomimetic molecules, and drugs of various chemical structures [14–17]. *S. pombe* and *S. cerevisiae*, two widely used model yeasts, have a single POT family protein named Ptr2 [18,19]. Recently, a novel fungal oligopeptide transporter (FOT) family, unrelated to the POT family, has been identified in some fungal species [20,21]. In *S. cerevisiae*, only the strains used for wine brewing contain this transporter, whereas laboratory strains lack the gene for the same [22].

In this study, we show that sensitivity to BlaS in yeasts depends on the presence of the POT family transporter Ptr2 [18,19,23]. Using a

Abbreviations: BlaS, blasticidin S; POT, proton-dependent oligopeptide transporter; FOT, fungal oligopeptide transporter.

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http://dx.doi.org/10.1016/j.bbamcr.2016.11.030
0167-4889/

yeast model system, we examined mouse-derived POT family protein PepT1, and a wine yeast-derived novel peptide transporter Fot1, for their role in the BlaS sensitivity. We have also examined the involvement of the bacterial POT family proteins in BlaS sensitivity in *E. coli*. Our findings suggest that di/tripeptide transporter Ptr2 imports BlaS into the yeast cells, and other POT family proteins in mouse and *E. coli* play a similar role.

2. Results

2.1. Ptr2 oligopeptide transporter is critical for BlaS sensitivity in yeasts

In the fission yeast *S. pombe*, the Ubr11 ubiquitin ligase stimulates the expression of the oligopeptide transporter Ptr2 [19] by degrading Upa1, a transcriptional repressor of the *ptr2* gene (Kitamura, unpublished). Since both *ubr11* and *ptr2* mutants are defective in the uptake of dipeptides, we tested whether the two mutants shared other phenotypes. We reported earlier that a mutant of the *ubr11* ubiquitin ligase in *S. pombe* showed weak resistance to some drugs such as inhibitors of ergosterol synthesis (terbinafine) or protein synthesis (hygromycin B and anisomycin) [24]. Unlike the *ubr11* mutant, the *ptr2* mutant is sensitive to hygromycin B, similar to the wild type strain (Fig. 1A). Interestingly, both *ptr2* and *ubr11* mutants were resistant to BlaS, another protein synthesis inhibitor (Fig. 1A and B). This resistant phenotype was suppressed by the ectopic expression of the Ptr2 from the heterologous *nmt* promoter in both mutants (Fig. 1A, compare second and third rows for *ubr11*Δ, and fourth and fifth rows for *ptr2*Δ), indicating that loss of Ptr2 expression was responsible for the BlaS resistance. When the BlaS sensitive wild type strain was cultured on BlaS containing agar medium, some BlaS resistant colonies appeared spontaneously. All the seven independent colonies, which were randomly chosen from ten such resistant colonies, failed to utilize dipeptide Ala-Gln as a sole nitrogen source, unlike the parental strain (data not shown). This observation further supported the close relationship between BlaS resistance and dipeptide uptake defect.

The simplest hypothesis that accounts for this finding is that BlaS is transported through Ptr2. Addition of soy peptides (di- and tripeptide-enriched mixture) to the medium abolished the sensitivity to BlaS (Fig. 1A), lending support to this hypothesis. In addition, the presence of an excess amount of naturally occurring dipeptides (Ala-Gln, Gly-Gly, Leu-Gly, Gly-Leu) mitigated the sensitivity to BlaS in the Ptr2 expressing strains (Fig. 1A and C), possibly by competing with BlaS in the uptake.

We examined whether Ptr2 was responsible for the uptake of BlaS in the evolutionarily distant yeast *S. cerevisiae*. Similar to *S. pombe*, *ptr2* mutants of *S. cerevisiae* could grow in the presence of BlaS (Fig. 2A). As expected, sensitivity in two different wild type strains was weakened in the presence of naturally occurring dipeptides (Fig. 2B), confirming that mitigation of BlaS toxicity by dipeptides was not a strain-specific effect. Unlike Gly-L-Leu, Gly-D-Leu was ineffective in suppressing the sensitivity. Contrasting effects of Gly-L-Leu and Gly-D-Leu were also observed in *S. pombe* (data not shown). Although D-amino acids are generally toxic to *S. cerevisiae*, Gly-D-Leu itself had no adverse effects for cell growth in both yeasts (data not shown), suggesting the unsuitability of Gly-D-Leu as a substrate for the Ptr2. Among the three *S. cerevisiae* strains tested, two laboratory strains (∑1278b and S288c) were sensitive to BlaS. However, EC1118, which is used for brewing wine, was resistant (Fig. 2C). We confirmed that both *PTR2* alleles of the intrinsically diploid EC1118 strain were inactive because of mutations (data not shown; [20]), further supporting the strong correlation between functionality of Ptr2 and BlaS sensitivity.

A POT family transporter is a proton-driven symporter. Since it uses the proton electrochemical gradient across the membrane to drive import, uptake of peptides is strongly affected by extracellular pH. The pH of unadjusted SD medium was 5.4 before starting the cell culture. High pH has a negative effect on yeast growth. However, the sensitivity to BlaS was significantly alleviated in the high pH (6.5 and 7.0) medium

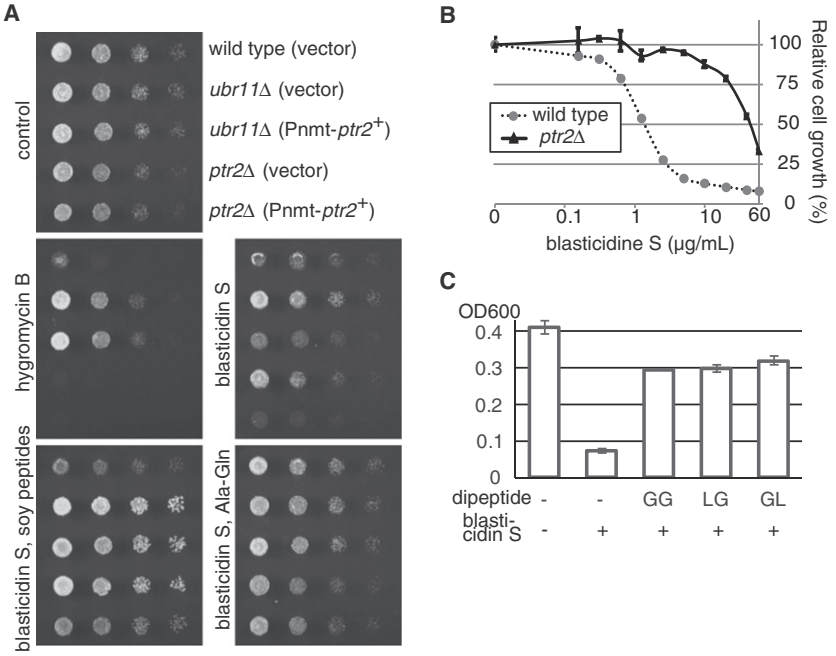

Fig. 1. Ptr2 is critical for BlaS sensitivity in fission yeast *S. pombe*. (A) Serially diluted *S. pombe* cells were spotted on the EMM medium containing hygromycin B (40 μg/mL), BlaS (6 μg/mL), soy peptides (0.1% *w*/*v*), and Ala-Gln (0.1% *w*/*v*). Strains: wild type (vector), KSP3168; *ubr11*Δ (vector), KSP3165; *ubr11*Δ (Pnmt-*ptr2*$^+$), KSP3166; *ptr2*Δ (vector), KSP3195; *ptr2*Δ (Pnmt-*ptr2*$^+$), KSP3196. (B) Wild type (L972) and *ptr2*Δ (KSP2422) strains were inoculated in liquid EMM containing BlaS at the indicated concentration. After 18 h, growth was monitored by measuring absorbance and expressed as percentage relative to BlaS-free culture. (C) Wild type (L972) cells were cultured in EMM containing BlaS (5 μg/mL) with or without indicated dipeptide (0.1% *w*/*v*). Amino acids in the dipeptides are L-forms and shown in single letter convention (e.g., LG means L-Leu-Gly). Absorbance (OD_{600}) was measured after 20 h.

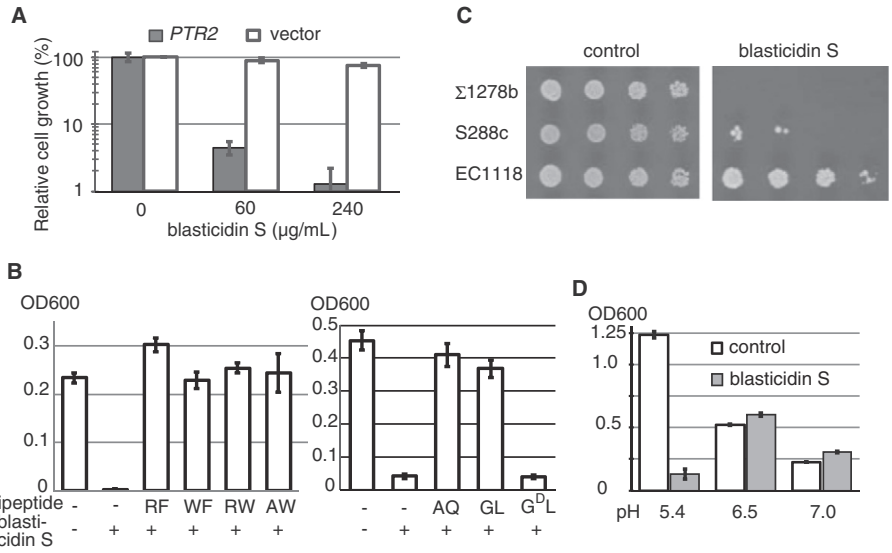

Fig. 2. Ptr2 is critical for BlaS sensitivity in budding yeast *S. cerevisiae*. (A) YPH499*ptr2*Δ strain was transformed with vector (YCplac33) or *PTR2* expressing plasmid (p*PTR2-3HA*). Each transformant was cultured in SD medium with or without BlaS. Cell growth was monitored after 20 h and expressed as in Fig. 1B. (B) S288c (left graph) and yA32 containing p*PTR2-3HA* (right graph) were cultured in liquid SD medium with BlaS (5 μg/mL) and dipeptide (0.1% *w*/*v*). Only G^DL (Gly-D-Leu) contains a D-amino acid, and the other six are L-form dipeptides. (C) Serially diluted cells of two laboratory strains (∑1278b and S288c) and one wine yeast strain (EC1118) were spotted on SD medium containing BlaS (30 μg/mL), or BlaS-free control medium. (D) yA32 containing p*PTR2-3HA* was cultured in pH-adjusted SD medium containing BlaS (10 μg/mL) for 20 h. Growth at each pH was compared with that in BlaS-free culture (control).

(Fig. 2D). Collectively, all these results indicate that Ptr2 is a portal for BlaS and is a major determinant of BlaS sensitivity in yeasts.

2.2. Uptake defects of dipeptides and those of BlaS are separable

We were interested in understanding the mechanism of the Ptr2 mediated uptake of BlaS. Within the structure of BlaS, a modified-arginine is linked to the deoxysugar acid via a peptide bond. This peptide-mimetic structure may play a role in the recognition of BlaS by Ptr2. We previously characterized many Ptr2 mutants of *S. cerevisiae*, which have amino acid substitutions in various regions of its structure [23]. In this study, we focused on five mutants, E92Q, R93K, K205R, W362L, and E480D, that showed the most severe dipeptide uptake defects. Non-functionality of these five Ptr2 mutants was confirmed by their inability to support the growth of the tryptophan-auxotrophic host strain when tryptophan was supplied in a dipeptide form, Arg-Trp (Fig. 3). Two *ptr2* mutants (E92Q and E480D) as well as the *ptr2*Δ null mutant (vector) were resistant to BlaS, consistent with the nonfunctional Ptr2, as expected. Unexpectedly, the other three mutants (R93K, K205R, and W362L) were sensitive to BlaS. Though the degree of growth defect varied among these mutants, sensitivity of the three Ptr2 mutants to BlaS indicated that they were still competent to import BlaS, despite the defect in the uptake of naturally occurring dipeptides.

2.3. Mammalian POT family transporter mediates BlaS uptake in yeast

Recently, novel FOT family transporters, which could import di/tripeptides, were identified in fungi, including in *S. cerevisiae* strains used in wine brewing [22]. In multicellular eukaryotes, import of di/tripeptides is primarily mediated by PepT1 and PepT2, representative members of the POT family [14]. Since the peptide transporters generally have broad substrate specificities, we next tested whether wine yeast-derived Fot1 and mouse PepT1 (mPepT1) transporters could also mediate uptake of BlaS. The *ptr2* mutant of laboratory *S. cerevisiae* strain was transformed with a plasmid expressing Fot1, mPepT1, or Ptr2 itself as a control. Functionality of these transporters was confirmed by their ability to support the auxotrophic strain's growth in the presence of naturally occurring dipeptides (Fig. 4A). Growth of the strains expressing peptide transporter was inhibited by high concentrations of BlaS (50 μg/mL) (Fig. 4B). However, the growth inhibitory effect of BlaS was not so severe in mPepT1 expressing strains, and only the Ptr2 expressing strain failed to grow even at 10-fold lower

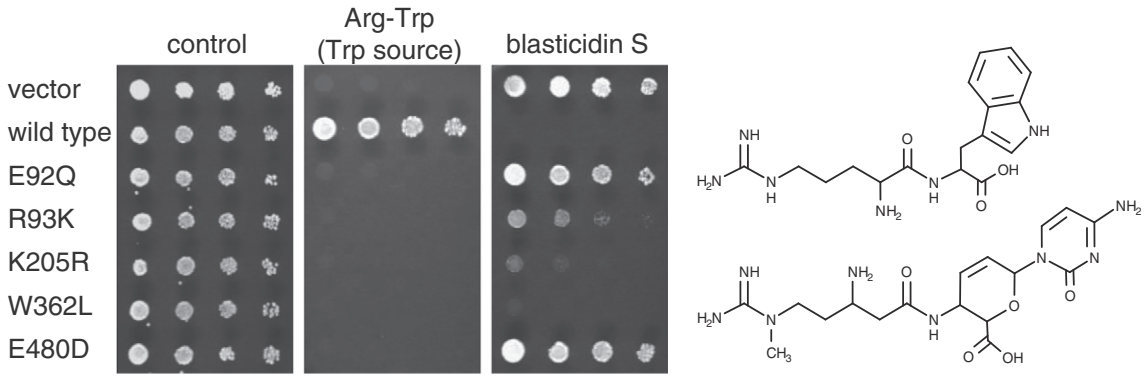

Fig. 3. Substrate-specific defects of the Ptr2 mutants. YPH499*ptr2*Δ was transformed with the plasmid expressing *PTR2* (either wild type or harboring indicated mutation), and spotted on SD medium after serial dilution. Tryptophan is essential for the growth of YPH499 and was supplied as monomeric tryptophan (left and right panels) or Arg-Trp dipeptide at 0.1 mM (middle panel). BlaS was added to a concentration of 40 μg/mL (right panel). Structures of Arg-Trp (top) and BlaS (bottom) are shown on the right.

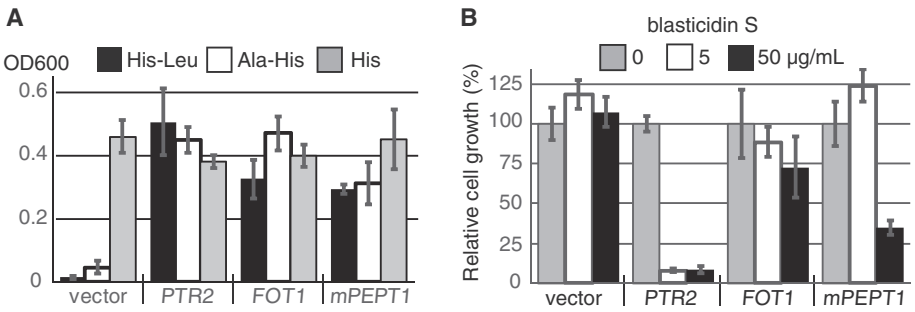

Fig. 4. Effect of wine yeast Fot1 and mouse PepT1 on BlaS sensitivity in yeast. YPH499*ptr2*Δ was transformed with either vector (YCplac33), p*PTR2-3HA*, pGK426(*FOT1*) or pGK426(*mPEPT1*). Note that *PTR2* was expressed with its own promoter from single copy YCp vector, whereas *FOT1* and *mPEPT1* were expressed with strong *PGK* promoter from multicopy YEp vector. (A) Fot1 and mPepT1 could substitute the function of Ptr2. YPH499 needs histidine for growth. Transformants with each plasmid were cultured in SD containing either dipeptide (His-Leu or Ala-His) or free amino acid (His) as a source of histidine. Dipeptide dependent growth was observed in strains expressing any one of the peptide transporters but not in the control strain (vector). (B) Cells were cultured in the presence of BlaS at 5 or 50 μg/mL concentration. Growth is expressed as percentage relative to the BlaS-free culture.

concentration (5 μg/mL). Therefore, peptide transporters in POT family could import BlaS; however, Ptr2 was the most potent in its import.

2.4. Bacterial POT family transporters potentiate BlaS sensitivity in E. coli

POT family transporters are conserved in prokaryotes. *E. coli* encodes four such members [DtpA (YdgR/TppB), DtpB (YhiP), DtpC (YjdL), and DtpD (YbgH)] in its genome [25–28]. Since BlaS inhibits the growth of prokaryotes also, we examined whether the involvement of POT proteins in BlaS sensitivity was conserved in *E. coli*. We tested the BlaS sensitivity of a strain containing a lac promoter-driven plasmid expressing one of the Dtp proteins. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) at a low concentration (7.5 μM) was used as an inducer because strong induction with high concentration (100 μM) significantly delayed the growth of cells harboring Dtp plasmids (data not shown). Growth was not affected at 7.5 μM (Fig. 5; comparing the growth profiles of vector and Dtp plasmid containing cells in the absence of BlaS). Even at this low level of induction, expression of POT family transporters, especially DtpA and DtpD, significantly enhanced the susceptibility to BlaS. Therefore, POT family peptide transporters may import BlaS in prokaryotes as well. BlaS exerted only a limited or no growth-inhibitory effect on strains expressing DtpB or DtpC, compared to those containing the control vector. Differences in the effects of different Dtp proteins may reflect the difference in their substrate specificities.

3. Discussion

BlaS is an antibiotic that inhibits protein synthesis, and is effective against both prokaryotes and eukaryotes. Though the mechanism of action by which it inhibits cell growth is well understood, knowledge of its cellular uptake mechanism is limited. In this study, we demonstrated that the presence of di/tripeptide transporter, Ptr2, of the POT family is necessary for sensitivity to BlaS in *S. pombe* and *S. cerevisiae*, two evolutionarily distinct yeast species. Presence of dipeptides in the medium interfered with the fungicidal action of BlaS. In addition, BlaS sensitivity was alleviated by alkalization of the medium. All these observations support the possibility that BlaS is imported through di/tripeptide transporter, Ptr2. Dipeptides competitively inhibited the uptake of BlaS through the same transporter. Extracellular alkalization had a negative effect on the cotransport of proton and imported molecule (dipeptide or BlaS), an essential feature of a POT family transporter. Since the two yeasts are distantly separated on the evolutionary tree, it is likely that import of BlaS through Ptr2 related oligopeptide transporter also occurs in other fungi and in higher eukaryotes. So far, two BlaS resistant mutations were reported in *S. cerevisiae* [29]. Though causative genes in these mutants have not been identified, it is likely that they are involved in the transcription, proper folding after translation, or membrane transport of Ptr2.

One significant finding in this study is that three mutant Ptr2 proteins differed in their response to naturally occurring dipeptides and

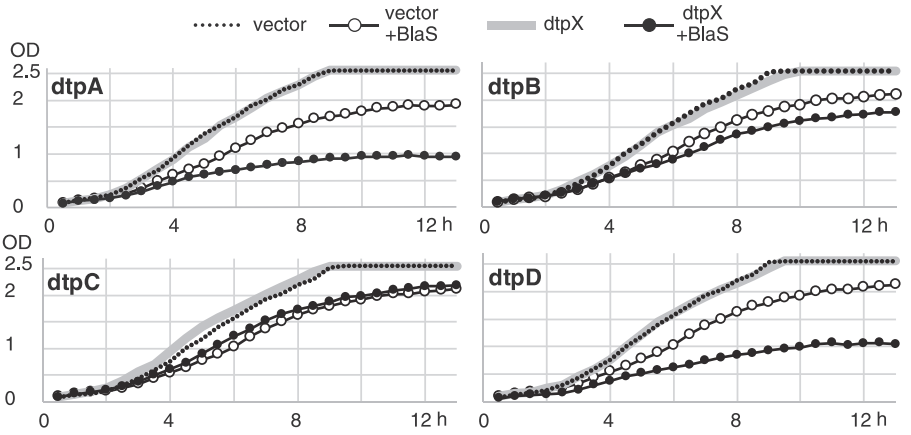

Fig. 5. Enhancement of BlaS sensitivity by bacterial POT family transporter in *E. coli*. The AG1 strain harboring vector (pCA24N, −gfp) or ASKA clone expressing one of Dtp proteins (DtpA-D) was cultured in LB for 13 h. Expression of Dtp proteins was induced by 7.5 μM of IPTG at 0 h. The culture was divided into two portions, and BlaS was added at 40 μg/mL concentration to one portion. Growth was continuously recorded every 30 min by measuring absorbance of the cultures.

BlaS. The five Ptr2 mutations of *S. cerevisiae* used in this study were unable to support dipeptide dependent growth, and the mutants were nonfunctional (Fig. 3, [23]). Consistent with this finding, two of the mutants (E92Q and E480D) were resistant to BlaS, similar to a *ptr2*Δ strain in which the *PTR2* gene was completely deleted. The three other mutants (R93K, K205R, and W362L) retained sensitivity to BlaS, indicating that the three Ptr2 mutants were not completely inactive. These five mutated residues constitute the substrate-binding pocket, and play a pivotal role in peptide recognition and/or coupling with proton [23]. In the structure of BlaS, methyl-arginine is linked to a glucuronic acid-derived hexose via a peptide bond. This peptidyl structure seems to be partly similar to that of the Arg-Trp dipeptide used in this study (Fig. 3). Our finding may indicate that Ptr2 recognizes and/or transports dipeptides and BlaS in a slightly different manner. For example, the residues in Ptr2 that are involved in binding BlaS seem to be different from those that bind naturally occurring dipeptides. To address these questions, it is important to know how Ptr2 recognizes BlaS, which can only be elucidated by detailed analyses like crystallographic structural study and molecular simulation. It is also essential to understand the mechanism of action of POT transporters with respect to various substrates, considering their broad specificity to a variety of substrates, ranging from a multitude of naturally occurring peptides to synthetic pharmaceutical drugs. We also showed that BlaS was not a good substrate for the wine yeast-derived Fot1, a peptide transporter of the recently identified novel FOT family, which also has broad peptide specificity [20]. Comparison of the substrate recognition and transport mechanisms between POT and FOT transporters provides an important insight for developing improved fungicidal drugs or yeast strains useful for industrial purposes such as fermentation.

It was reported that uptake of BlaS was mediated by Leucine-rich repeat-containing 8 (LRRC8) proteins in mammalian cells [8]. The LRRC8 proteins are not conserved in lower eukaryotes and prokaryotes; however, BlaS inhibits cell growth in all eukaryotes and also in prokaryotes. These organisms should have another route for cellular entry of BlaS. Obviously, the candidate is POT family peptide transporter. We showed that mouse-derived PepT1 di/tripeptide transporter was able to import BlaS, when it was expressed in yeast. The effect of mPepT1 was not remarkable, which might be due to the use of a heterologous expression system. We used the mouse-derived cDNA. Therefore, its codon usage was quite different from that in yeast. It is also critical that the translated mouse protein is properly folded and transported to the cell surface in yeast. Note that Fot1 was derived from wine strain of *S. cerevisiae* itself. Considering the functional conservation of POT family proteins, it is possible that BlaS is transported by PepT1 and/or PepT2 in higher eukaryotes also. However, PepT1 and PepT2 are not universally expressed in all tissues. Depending on the tissue type, BlaS may use multiple transport mechanisms in mammals.

In prokaryotes such as *Pseudomonas aeruginosa* PA14 strain, the ABC transporter NppA1A2BCD is required for the uptake of nucleoside antibiotics including BlaS [30]. However, unlike yeast *ptr2* mutants, an *E. coli* strain lacking yejABEF, the closest homolog of NppABCD, was not resistant to BlaS [30]. In addition, introduction of an NppA1A2BCD-expressing plasmid in *E. coli* did not alter the BlaS sensitivity, although the same plasmid effectively lowered the minimum inhibitory concentration of two other peptidyl nucleoside drugs, pacidamycin D and albomycin [30]. This indicates that the involvement of the NppABCD/YejABEF in the uptake of BlaS may be species-specific. We showed in *E. coli* that POT family proteins, especially DtpA and DtpD could mediate the uptake of BlaS. However, BlaS sensitivity of an *E. coli* quadruple POT mutant, which lacked all four *dtpA/B/C/D* genes (4dtpΔ), or even the 4dtpΔ yejABEF quintuple mutant strain was comparable to that of the parental wild type strain (data not shown). In addition to the POT family Dtp proteins, *E. coli* uses prokaryote-specific ABC transporters, Dpp and Opp systems, for the import of dipeptides [14]. If the peptidyl nature of BlaS is essential for the recognition and import by transporters, Dpp and Opp systems may also support uptake of BlaS in *E. coli*.

Table 1
Yeast strains used in this study.

Name	Genotype
(S. pombe)	
KSP2422	h^+ *ptr2*::*ura4*$^+$ *ura4-C190T*
KSP3165a	h^+ *ubr11*::*kanMX6 leu1–32*::[*leu1*$^+$; *nmt41*(*HFF*)]$_{int}$
KSP3166a	h^+ *ubr11*::*kanMX6 leu1–32*::[*leu1*$^+$; *nmt41*(*HF-ptr2*$^+$)]$_{int}$
KSP3168	h^- *leu1–32*::[*leu1*$^+$; *nmt41*(*HFF*)] $_{int}$
KSP3195	h^- *ptr2*::*ura4*$^+$ *ura4-D18 leu1–32*::[*leu1*$^+$; *nmt41*(*HFF*)] $_{int}$
KSP3196	h^- *ptr2*::*ura4*$^+$ *ura4-D18 leu1–32*::[*leu1*$^+$; *nmt41*(*HF-ptr2*$^+$)] $_{int}$
L972	h^- (wild type, prototroph)
(S. cerevisiae)	
EC1118[1)]	(wine yeast strain, diploid, prototroph)
S288c[2)]	(laboratory strain, haploid, prototroph)
∑1278b[2)]	(laboratory strain, haploid, prototroph)
yA32[3)]	*MAT***a** *ptr2*::*kanMX6 ura3–52* (S288c-derived)
YPH499	*MAT***a** *his3-Δ200 leu2-Δ1 lys2–801 trp1-Δ1 ade2–101 ura3–52*
YPH499*ptr2*Δ	*ptr2*::*kanMX* in YPH499

Sources: 1) T. Fujii (NRIB, Japan), 2) H. Takagi (NAIST, Japan), 3) A.V. Melnykov (WUSM, USA). Other strains are our laboratory stocks.

In conclusion, our data shows that peptide transporter is one of the key molecules that mediates the uptake of BlaS in both prokaryotes and eukaryotes. Further analysis of the detailed mechanism of recognition and import of BlaS by POT proteins will help to understand the molecular basis of the broad substrate specificities of versatile peptide transporters.

4. Materials and methods

4.1. Strains, media, and cell culture

Yeast strains used in this study are summarized in Table 1. Edinburgh minimal medium (EMM) for *S. pombe* [31] and synthetic dextrose medium (SD) for *S. cerevisiae* [32] were used as minimal media, with appropriate supplements (nucleotide bases and amino acids). Initial pH of unbuffered SD medium was 5.4. pH was not adjusted unless specified. To prepare high pH medium, SD medium was buffered with 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) and 50 mM 2-morpholinoethanesulfonic acid (MES), and the pH was adjusted to 6.5 or 7.0 with NaOH [33]. The *E. coli* strain, AG1, was cultured in LB medium. Blasticidin S was purchased from InvivoGen and Wako Pure Chemical Industries Ltd. Dipeptides were purchased from Kokusan chemical Co. Ltd., PEPTIDE INSTITUTE INC., Tokyo chemical industry Co. Ltd., Bachem, and Sigma-Aldrich. Soy peptide (HINUTE HK) was kindly provided by Ms. Kitagawa (Fuji Oil Co. Ltd.). Cells were cultured in liquid medium in 96 well plates without shaking, or in a small test tube with shaking. To monitor cell growth, absorbance of the cell culture was measured by spectrophotometry or continuously recorded with OD-Monitor C&T (TAITEC Corporation). All experiments were repeated at least twice, and the representative results are shown. Data are shown as mean ± SD of at least three technical replicates for each sample.

4.2. Plasmids

Plasmids expressing Ptr2 in *S. pombe* or in *S. cerevisiae* (pDUAL41-HF-*ptr2*$^+$ and p*PTR2-3HA*, respectively) have been described [19,23]. Plasmids expressing Ptr2 mutants (E92Q, R93K, K205R, W362L, and E480D) in *S. cerevisiae* were also used [23]. The pGK426 vector was used to express the wine yeast-derived Fot1 or the mouse-derived PepT1 in *S. cerevisiae*. This episomal, multi-copy vector enables the expression of an inserted gene from the strong *PGK* promoter [34]. pGK426 was obtained from the National Bio-Resource Project (NBRP)-Yeast, Japan. For Fot1, the protein-coding region was amplified by PCR from the genomic DNA of EC1118 strain [20–22]. The mouse PepT1 cDNA was obtained from DNAFORM, and the protein-coding region was amplified by PCR. Amplified DNA was inserted in the *Bam*HI site

of the pGK426 vector. The ASKA (−gfp) clones [35] expressing *E. coli* POT family Dtp protein were obtained from NBRP-*E. coli* (NIG, Japan).

Author contributions

KK conceived and designed the research; KK, EZBK and FA performed experiments; KK analyzed the data and wrote the manuscript.

Competing financial interests

The authors declare that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

We would like to thank Drs. Takagi (Nara Institute of Science and Technology), Fujii (National Research Institute of Brewing) and Melnykov (Washington University School of Medicine) for providing yeast strains, and Drs. Tanaka and Yamashita (Hiroshima University) for the discussions. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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