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Peptidoglycan-associated outer membrane protein Mep45 of rumen anaerobe *Selenomonas ruminantium* forms a non-specific diffusion pore via its C-terminal transmembrane domain

Seiji Kojima[1,2,*], Kanako Hayashi[3], Saeko Tochigi[2], Tomonobu Kusano[2], Jun Kaneko[3] and Yoshiyuki Kamio[3,4]

[1]*Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Sendai, Japan;* [2]*Graduate School of Life Sciences, Tohoku University, Sendai, Japan;* [3]*Graduate School of Agricultural Science, Tohoku University, Sendai, Japan;* [4]*Graduate School of Comprehensive Human Sciences, Shokei-Gakuin University, Natori, Japan*

The major outer membrane protein Mep45 of *Selenomonas ruminantium*, an anaerobic Gram-negative bacterium, comprises two distinct domains: the N-terminal S-layer homologous (SLH) domain that protrudes into the periplasm and binds to peptidoglycan, and the remaining C-terminal transmembrane domain, whose function has been unknown. Here, we solubilized and purified Mep45 and characterized its function using proteoliposomes reconstituted with Mep45. We found that Mep45 forms a nonspecific diffusion channel via its C-terminal region. The channel was permeable to solutes smaller than a molecular weight of roughly 600, and the estimated pore radius was 0.58 nm. Truncation of the SLH domain did not affect the channel property. On the basis of the fact that Mep45 is the most abundant outer membrane protein in *S. ruminantium*, we conclude that Mep45 serves as a main pathway through which small solutes diffuse across the outer membrane of this bacterium.

Key words: outer membrane; rumen; channel; porin; Gram-negative bacteria

Selenomonas ruminantium, an obligate anaerobe ubiquitously found in rumen, has a typical Gram-negative-type cell surface structure comprising cytoplasmic membrane, peptidoglycan, and outer membrane[1,2] but is distinguished from conventional model organisms of Gram-negative bacteria, such as *Escherichia coli* or *Salmonella typhimurium,* by (i) the absence of murein–lipoprotein and Tol–Pal complex,[2] which are essential components for anchoring the outer membrane to peptidoglycan,[3–6] and (ii) the presence of cadaverine covalently bound to the α-carboxyl group of the D-glutamic acid residue of peptidoglycan.[7,8] Our previous reports showed that cadaverine is required for the binding of major outer membrane protein Mep45 of *S. ruminantium* to the peptidoglycan, and the Mep45-peptidoglycan interaction constitutes the anchor between the outer membrane and peptidoglycan, thereby maintaining the stability and integrity of the outer membrane of this bacterium.[9–11] The presence of peptidoglycan-bound polyamines and homologs of Mep45 is a well-conserved feature of bacteria belonging to the class *Negativicutes*,[12] a group that is known for possessing a Gram-negative cell surface structure despite being classified into the phylum *Firmicutes* (Gram-positive bacteria) by nucleotide sequence-based phylogenetic analysis.[13]

Mep45 comprises an N-terminal S-layer homologous (SLH) domain (A^1 to K^{68}) that protrudes into the periplasmic space and binds to peptidoglycan, and a C-terminal transmembrane region (L^{69} to F^{409})[9] (Fig. 1(A)). Mep45 is the most abundant outer membrane protein (approximately 3×10^6 molecules per cell) in *S. ruminantium* and is roughly estimated to cover more than 80% of the cell surface area.[9] Thus, the properties of the outer membrane of this bacterium are expected to be directly associated with the function of the Mep45 C-terminal region. Considering the facts that *S. ruminantium* grows almost as fast as *E. coli* (doubling time ~25 min) when it is cultured in an optimized medium,[10] and that it is also one of the fastest-growing bacteria in the rumen,[14] the permeability of the outer membrane is supposed to be reasonably high because of the necessity of ensuring a sufficient speed of influx of the nutrients across the outer membrane to sustain the rapid cell propagation. In fact, Mep45 was expected to form a β-barrel structure with a probable pore-forming activity, on the basis of its amino acid sequence and biochemical analysis.[9]

Here, we purified Mep45 in a solubilized form and characterized its function using proteoliposomes reconstituted with Mep45. Mep45 forms a non-specific diffusion pore in its C-terminal region. Together with our

(A) SLH domain

NH$_2$ — [ ] Transmembrane region [ ] — COOH

(B)

2,000 669 200 150 60 (kDa)

(C) M   **(D)** M

180   180
140   140
100   100
75   75
60   60
45   ◀ Mep45   45
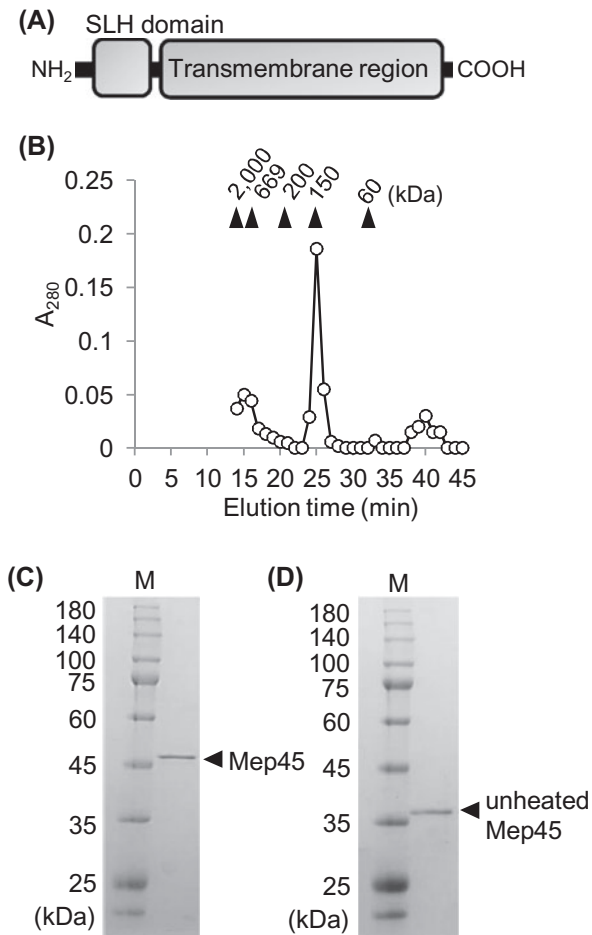35   35   ◀ unheated Mep45
25   25
(kDa)   (kDa)

Fig. 1. Purification of Mep45.

Notes: (A) Domain arrangement of Mep45. Mep45 comprises an N-terminal SLH domain and C-terminal transmembrane region. (B) Gel-filtration chromatography of Mep45. Molecular mass standards: Blue dextran (2000 kDa), thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and albumin (66 kDa). (C, D) SDS-PAGE of purified Mep45. The sample in panel C was heat-denatured by incubating at 100 °C for 5 min. In panel D, this heat-denaturing procedure was omitted. The gels were stained with Coomasie Brilliant Blue (CBB). M, molecular mass standards.

previous report and the data presented herein, we conclude that Mep45 is a bifunctional protein that acts by its C-terminal region as a main pathway through which solutes diffuse across the outer membrane, and that it fulfills a role in stabilizing the outer membrane by its periplasm-exposed SLH domain that binds to peptidoglycan.

Materials and methods

Strain and culture conditions. *S. ruminantium* subsp. *lactilytica* TAM6421 was cultured in TYG medium at 37 °C, as described previously.[9]

Purification of Mep45 and SLH-domain truncated Mep45 (ΔSLH-Mep45). Cells were harvested from 250 ml of culture and suspended in 20 mM Tris-HCl (pH 7.5) (Tris buffer). Cells were disrupted by sonication (30-s pulse and 30-s rest, eight times), and unbroken cells were removed by centrifugation at 2500 × *g* for 5 min. The crude envelopes were pelleted

by centrifugation at 100,000 × *g* for 1 h at 4 °C. The crude envelopes were suspended in 0.5% lauroylsarcosine [critical micelle concentration (CMC) ≈ 0.43%] in Tris buffer and incubated for 15 min at room temperature (RT). This treatment extracts the majority of membrane proteins other than Mep45. The insoluble fraction containing Mep45 as the dominant protein was pelleted by centrifugation at 20,000 × *g* for 30 min at RT. To solubilize Mep45, this insoluble material was suspended in 2% lithium dodecyl sulfate (LDS) (CMC ≈ 0.23%) in Tris buffer containing 0.5 M LiCl and 10 mM EDTA, and incubated at 37 °C for 15 min. Supernatant was collected after centrifugation at 20,000 × *g* for 30 min at RT. Solubilized Mep45 was further purified by gel-filtration high-performance liquid chromatography (HPLC). HPLC conditions were as follows: column, Superdex 200 Increase 10/300 GL (GE Healthcare, Buckinghamshire, UK); eluent, 0.1% LDS in 10 mM Tris-HCl (pH 7.5) containing 0.4 M LiCl; flow rate, 0.5 ml/min.

To prepare SLH domain-truncated Mep45 (ΔSLH-Mep45), the crude envelopes were digested by 100 μg/ml trypsin in Tris buffer at 37 °C for 30 min. After incubation, the envelopes were collected by centrifugation at 20,000 × *g* for 20 min at RT. After a second round of trypsin digestion, ΔSLH-Mep45 was solubilized and purified by the same protocol for intact Mep45 as described previously. The purity of ΔSLH-Mep45 was determined by densitometry of protein bands on a SDS-PAGE gel using ImageJ software.

Quantification of Mep45-bound lithium dodecyl sulfate (LDS). LDS was quantified according to the method by Hayashi.[15]

Liposome swelling assay. Before reconstituting into liposomes, Mep45 (or ΔSLH-Mep45) preparation was dialyzed overnight at 4 °C against 0.5 mM Tris-HCl (pH 7.5) to remove excess LiCl. To examine the channel-forming activity, Mep45 was reconstituted into liposome comprising 2.4 μmol phosphatidylcholine and 0.15 μmol dicetylphosphate. The experimental procedure was the same as described by Nikaido et al.[16] The proteoliposome suspension was mixed with solutes to be tested in an isotonic condition, and proteoliposome swelling caused by the influx of solutes was monitored by measuring the absorbance at 400 nm (OD$_{400}$). To examine the permeation of various solutes including mono- or di-anionic compounds, we followed the protocol described by Nikaido and Rosenberg.[17] Briefly, 3 to 10 μg of Mep45 (or ΔSLH-Mep45) protein was reconstituted into the liposomes comprising 6.2 μmol phosphatidylcholine and 0.2 μmol dicetylphosphate. Proteoliposomes were suspended in 1 mM NAD-imidazole buffer (pH 6.1) containing 4 mM sodium NAD and 12 mM stachyose. The reaction was initiated by diluting 17 μl of proteoliposome suspension into 600 μl of reaction mixture, whose composition was as follows: 1 mM sodium NAD, solutes to be tested (18 mM for nonelectrolytes, 9 mM for sodium salt of monoanionic compounds, or 6 mM for disodium salt of dianionic compound), and 1 mM

NAD-imidazole (pH 6.1). The swelling of proteoliposome was monitored by measuring OD_{400}.

Results

Solubilization and purification of Mep45

Previous research showed that Mep45 is resistant to mild detergent treatments.[9] We therefore first isolated Mep45 as an insoluble state by extracting membrane proteins other than Mep45 with 0.5% lauroylsarcosine, prior to searching for the conditions that solubilized Mep45. We found that 2% lithium dodecyl sulfate (LDS) containing 0.5 M LiCl and 10 mM EDTA, which destabilizes lipopolysaccharide (LPS) by chelating LPS-associated Mg^{2+}, was effective in solubilizing Mep45 at 37 °C. More than 90% of Mep45 protein was solubilized by this treatment. We further purified Mep45 by gel-filtration chromatography (Fig. 1(B)). Mep45 eluted at the fraction close to alcohol dehydrogenase, whose molecular mass is 150 kDa. Because the solubilized membrane protein was expected to bind a substantial amount of LDS,[18] we quantified the Mep45-bound LDS and determined that solubilized Mep45 contains 0.92 ± 0.09 μg of LDS per μg of protein. Thus, the estimated molecular mass of solubilized Mep45 is around 78 kDa, which roughly corresponds to that of a Mep45 dimer (apparent molecular mass of undenatured Mep45 is 36 kDa as judged by SDS-PAGE; see below). This was consistent with our previous cross-linking study, which detected a dimeric form of Mep45 in an outer membrane preparation.[9] However, in a solubilized state, this probable dimer seemed only loosely associated with each protomer, as SDS-PAGE analysis of undenatured Mep45 detected only the monomeric form (Fig. 1(C)). A cross-linking experiment using formaldehyde, glutaraldehyde, or disuccinimidyl suberate, unfortunately, produced only high-molecular-weight aggregate that could not enter the acrylamide gel (data not shown). Purified Mep45 showed different mobility in SDS-PAGE analysis dependent on the presence or absence of heat-denaturing treatment before electrophoresis (Fig. 1(C, D)). Undenatured Mep45 migrated faster than heat-denatured Mep45 and showed an apparent molecular mass of 36 kDa. This "heat-modifiability" is a well-known property of some β-barrel outer membrane proteins[18,19] and suggests that our solubilization and purification procedure did not cause any apparent denaturation of the Mep45 protein.

Mep45 is a channel-forming protein

We examined the function of Mep45 using proteoliposomes reconstituted with purified Mep45. We first examined the permeation of small carbohydrates into the proteoliposomes, by monitoring the swelling of proteoliposome caused by the influx of these solutes that results in the reduction in the absorbance (OD_{400}) of the reaction mixture (Fig. 2(A)). Rapid swelling was observed upon mixing the proteoliposome with arabinose, glucose and *N*-acetylglucosamine (GlcNAc), indicating that Mep45 forms a channel permeable to these compounds. Raffinose, on the other hand, did not cause detectable swelling. We then examined the
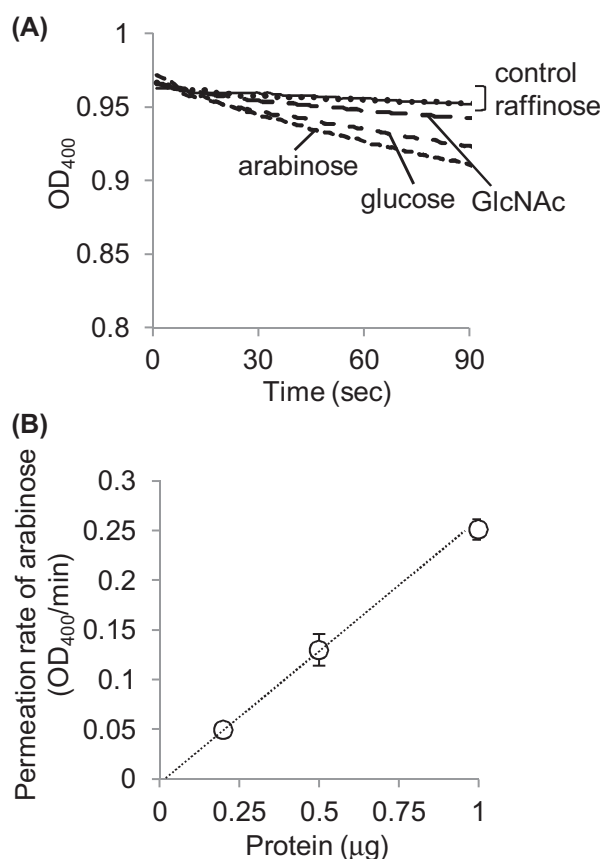

Fig. 2.   Channel-forming activity of Mep45.
  Notes: (A) Liposome-swelling assay of proteoliposome reconstituted with purified Mep45 (0.4 μg of protein). GlcNAc, *N*-acetylglucosamine. (B) Channel-forming efficiency of Mep45. Varying amounts of Mep45 were reconstituted into liposomes, and the permeation rate of arabinose was measured. Data represent the mean value of three independent experiments ± SD.

channel-forming efficiency of Mep45 by examining the permeability of arabinose against the proteoliposome with various amount of Mep45 protein (Fig. 2(B)). A linear correlation between the permeability and the amount of reconstituted Mep45 protein was observed, and we found that the permeation rate produced by 1 μg of Mep45 protein was comparable to that produced by the porin channel of *E. coli*.[20] We confirmed that liposomes reconstituted with the outer membrane preparation of *S. ruminantium*, which contains Mep45 as a dominant protein, showed similar permeability to the liposomes reconstituted with purified Mep45 (data not shown). This suggests that procedures for purifying and reconstituting Mep45 did not affect its function significantly and is of importance because we used the liposomes made of phosphatidylcholine and dicetylphosphate, despite that the dominant phospholipid in the *S. ruminantium* outer membrane is phosphatidylethanolamine.[1]

Channel properties of Mep45

We further characterized the channel properties of Mep45 by testing the permeability of various solutes (Fig. 3(A)). The permeability was predominantly dependent on solute size (molecular weight), whereas
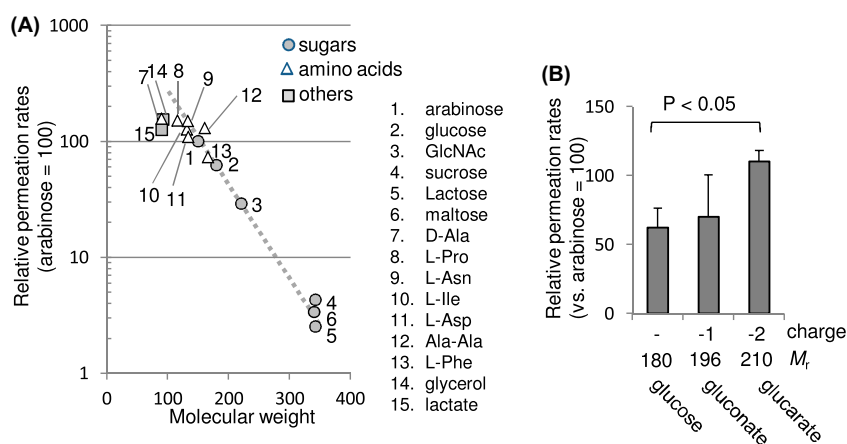
Fig. 3.   Channel properties of Mep45.

Notes: (A) Relative permeation rates of various solutes through the Mep45 channel (permeation rate of arabinose = 100). GlcNAc, *N*-acetylglu-cosamine. Data represent the mean value of three independent experiments. (B) Relative permeation rates of glucose, gluconate, and glucarate. Data represent the mean value of three independent experiments. The statistical significance of the data was evaluated by Student's *t*-test. M_r, molecular weight.
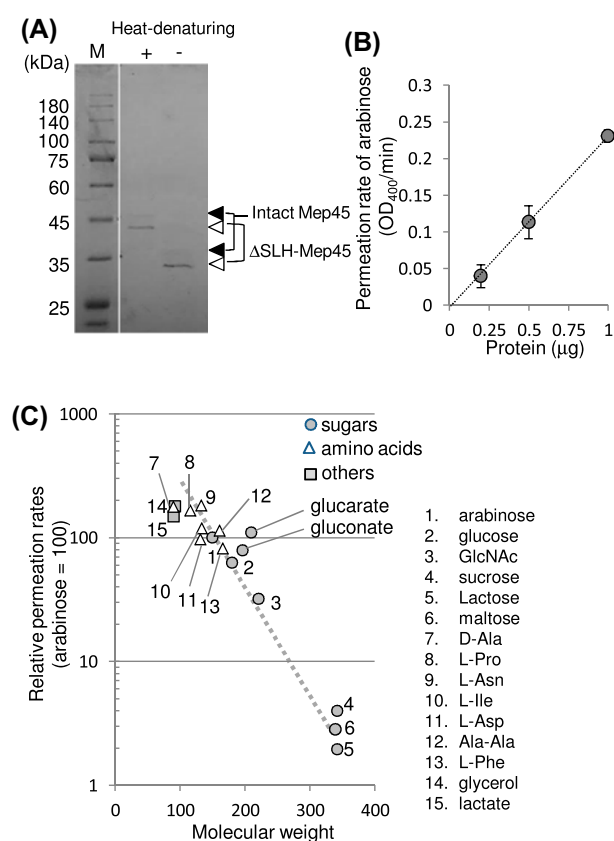

Fig. 4.   Characterization of ΔSLH-Mep45.

Notes: (A) SDS-PAGE of purified ΔSLH-Mep45. The sample was subjected (or not subjected) to heat denaturation by incubating the sample at 100 °C for 5 min. The gel was stained with CBB. (B) Channel-forming efficiency of ΔSLH-Mep45. Proteoliposomes were reconstituted with various amounts of ΔSLH-Mep45, and the permeation rate of arabinose was measured. Data represent the mean value of three independent experiments ± SD. (C) Relative permeation rates of various solutes through the ΔSLH-Mep45 channel (permeation rate of arabinose = 100). Data represent the mean value of three independent experiments.
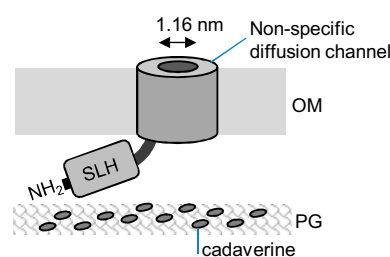

Fig. 5.   Schematic representation of Mep45 in the outer membrane of *S. ruminantium*.

Notes: Mep45 binds to peptidoglycan by its SLH domain. This interaction requires peptidoglycan-bound cadaverine. The C-terminal region of Mep45 forms a non-specific diffusion channel, with an estimated pore diameter of 1.16 nm. PG and OM, peptidoglycan and outer membrane, respectively.

no obvious substrate specificity was observed. This indicates that Mep45 forms a non-specific diffusion pore, and thus, Mep45 can be classified as a "classical porin", such as OmpF of *E. coli*.[21] The pore size can be theoretically estimated using the experimentally determined permeation rates of solutes, molecular radii of the solutes, and their diffusion coefficients in water, according to the analysis of the *E. coli* porin channel carried out in 1981[20] and 1983.[17] We estimated the pore radius of Mep45 to be around 0.58 nm, which is the same as OmpF. Porin channels in *E. coli* act principally as non-specific diffusion pores, nonetheless, the permeation rates of solutes are affected by their charge and by the electrostatic profile of the pore lining.[17,22,23] In this regard, it was of interest to examine the permeation rates of negatively charged solutes through Mep45, because Mep45 has a greater number of basic amino acid residues (the calculated pI of the C-terminal region is 9.4) compared to OmpF (pI = 4.4) or OmpC (pI = 4.3), whose pore retards the diffusion of solutes with negative charge(s).[17] Therefore, we compared the permeation rates of glucose and its monoanionic and dianionic derivatives, gluconate (pK_a = 3.7) and glucarate (saccharate) (pK_a = 3.0), respectively. The permeation rate of glucarate was facilitated by nearly 2-fold compared to that of glucose, whereas only a slight facilitation was observed between glucose and gluconate (Fig. 3(B)). We also examined the permeation of lactate and aspartate, and compared it to glycerol and asparagine, respectively, but no

obvious facilitation was observed (Fig. 3(A)). The facilitation of glucarate permeation was notable because this compound is larger than glucose by a molecular weight of 30, and this difference should be enough to produce a significant retardation of the permeation of nonelectrolytes, as apparent by the difference observed between arabinose and glucose, or glucose and GlcNAc. These results suggest that the Mep45 channel, in contrast to OmpF or OmpC, facilitates the diffusion of dianionic compounds.

Channel properties of Mep45 are unaffected by truncation of its SLH domain

The pore-forming activity of Mep45 is expected to be attributed to its C-terminal region. To affirm this expectation and clarify whether the SLH domain and the C-terminal region are functionally independent of each other, we prepared SLH domain-truncated Mep45 by trypsin-treatment (ΔSLH-Mep45; corresponds to L[69] to F[409] of intact Mep45)[9] (Fig. 4(A)). ΔSLH-Mep45 was solubilized in the same conditions as intact Mep45, and purified by gel-filtration chromatography. It eluted about 2 min later than the intact Mep45 (data not shown). ΔSLH-Mep45 showed heat-modifiability in SDS-PAGE (Fig. 4(A)). It should be noted, however, that unfortunately we were not able to completely remove the intact Mep45, and the ΔSLH-Mep45 preparation was contaminated with intact Mep45 protein by approximately 5%, as judged from densitometric analysis of the protein bands on SDS-PAGE gel (Fig. 4(A)). Nevertheless, since the majority (95%) of Mep45 in this preparation was SLH-domain-less, we considered this preparation is at least applicable to the experiment for assessing the effect of truncating the SLH domain on the channel properties of Mep45. We examined the channel-forming efficiency and substrate specificity of ΔSLH-Mep45. Results were almost identical to those of intact Mep45 (Fig. 4(B, C)). Thus, we concluded that the channel-forming activity indeed attributes to the C-terminal region and that the SLH domain does not functionally interact with the channel.

Discussion

The properties and permeability of the outer membrane of Gram-negative bacteria fundamentally affect the survival of the cell and its physiology by determining the flux of substances into the cell.[21] Mep45 has been considered to be central to the function of the outer membrane in *S. ruminantium*. In this study, we demonstrated that Mep45 forms a non-specific diffusion channel in its C-terminal region, with an estimated pore radius of 0.58 nm (Fig. 5). Mep45 is categorized as a "classical porins"[21] and represents the first example of an experimentally characterized porin channel in *Negativicutes*.

The permeation rate of a solute through the Mep45 channel is predominantly dependent on the size of the molecule; permeation rates of disaccharides were only 1 to 3% of that of arabinose, and rafinose, whose

Stokes radius (0.57 nm)[24] is almost the same as the estimated channel radius, did not pass through the channel at a detectable rate (Figs. 2, 3). On the basis of these observations, the molecular size of raffinose (molecular weight = 595) is expected to closely correspond with the exclusion limit of the Mep45 pore. Considering the fact that Mep45 is the most abundant protein of the outer membrane of *S. ruminantium*, the Mep45 channel likely serves as a main pathway for the uptake of small nutrients across the outer membrane *in vivo*. In this regard, it is noteworthy that the Mep45 channel allows the diffusion of anionic compounds at rates comparable (or higher in the case of dianionic compounds) to those without negative charge(s) (Fig. 3), contrary to many diffusion channels that are constitutively expressed in conventional Gram-negative model bacteria, such as *E. coli* and *S. typhimur-ium*.[17,21] Thus, the outer membrane of *S. ruminantium* is considered to be highly permeable to small solutes (presumably smaller than a molecular weight of roughly 600) regardless of the presence of negative charge(s). This property appears suitable for *S. ruminantium* to proliferate in the rumen, because this bacterium generally feeds on the hydrolyzed products of plant cell wall-associated polysaccharides such as small sugars[25] or its fermented products such as lactate, provided by other ruminal bacteria.[14] These nutrients are small and often acidic. It should be noted, however, that the *S. ruminantium* genome possesses six paralogs of Mep45,[26] among which the amino acid composition of the predicted C-terminal transmembrane domain differs substantially. Three of them are basic proteins similar to Mep45 (calculated pI = 8.7–9.8), but the other three are acidic (pI = 5.7–6.7). Thus, it is possible that *S. ruminantium* changes its outer membrane properties by regulating the expression of Mep45 paralogs, although the expression level of these paralogs in our laboratory condition is undetectably low. Environmental conditions that affect the expression of Mep45 paralogs is the topic required to be elucidated for further understanding of the outer membrane properties of *S. ruminantium*.

Author contribution

S.K. designed research, S.K., K.H., and S.T. performed the experiments, S.K., K.H., J.K., T.K., and Y.K. analyzed data, and S.K. wrote the manuscript. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

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