Bacillus subtilis Intramembrane Protease RasP Activity in *Escherichia coli* and *In Vitro*

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ABSTRACT RasP is a predicted intramembrane metalloprotease of *Bacillus subtilis* that has been proposed to cleave the stress response anti-sigma factors RsiW and RsiV, the cell division protein FtsL, and remnant signal peptides within their transmembrane segments. To provide evidence for direct effects of RasP on putative substrates, we developed a heterologous coexpression system. Since expression of catalytically inactive RasP E21A inhibited expression of other membrane proteins in *Escherichia coli*, we added extra transmembrane segments to RasP E21A, which allowed accumulation of most other membrane proteins. A corresponding active version of RasP appeared to promiscuously cleave coexpressed membrane proteins, except those with a large periplasmic domain. However, stable cleavage products were not observed, even in *clpP* mutant *E. coli*. Fusions of transmembrane segment-containing parts of FtsL and RsiW to *E. coli* maltose-binding protein (MBP) also resulted in proteins that appeared to be RasP substrates upon coexpression in *E. coli*, including FtsL with a full-length C-terminal domain (suggesting that prior cleavage by a site 1 protease is unnecessary) and RsiW designed to mimic the PrsW site 1 cleavage product (suggesting that further trimming by extracytoplasmic protease is unnecessary). Purified RasP cleaved His₆-MBP-RsiW(73–118) *in vitro* within the RsiW transmembrane segment based on mass spectrometry analysis, demonstrating that RasP is an intramembrane protease. Surprisingly, purified RasP failed to cleave His₆-MBP-FtsL(23–117). We propose that the lack of α -helix-breaking residues in the FtsL transmembrane segment creates a requirement for the membrane environment and/or an additional protein(s) in order for RasP to cleave FtsL.

IMPORTANCE Intramembrane proteases govern important signaling pathways in nearly all organisms. In bacteria, they function in stress responses, cell division, pathogenesis, and other processes. Their membrane-associated substrates are typically inferred from genetic studies in the native bacterium. Evidence for direct effects has come sometimes from coexpression of the enzyme and potential substrate in a heterologous host and rarely from biochemical reconstitution of cleavage *in vitro*. We applied these two approaches to the *B. subtilis* enzyme RasP and its proposed substrates RsiW and FtsL. We discovered potential pitfalls and solutions in heterologous coexpression experiments in *E. coli*, providing evidence that both substrates are cleaved by RasP *in vivo* but, surprisingly, that only RsiW was cleaved *in vitro*, suggesting that FtsL has an additional requirement.

KEYWORDS *Bacillus subtilis*, FtsL, RasP, RsiW, anti-sigma factor, cell division, endospores, intramembrane proteolysis, proteases, sporulation

Intramembrane proteases (IPs) are polytopic membrane proteins with active-site residues in transmembrane segments (TMSs). IPs cleave their substrates within a TMS or near the membrane surface, and regulated intramembrane proteolysis (RIP) controls diverse signaling pathways in a wide variety of organisms (1, 2). There are four types of

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FIG 1 Model of the σ^W activation pathway. Envelope stress sensed by PrsW causes it to cleave the extracytoplasmic domain of the anti- σ factor RsiW. The C-terminal end of RsiW is further degraded by one or more bulk proteases of unknown identity. This is proposed to allow RasP to cleave the TMS of RsiW. The N-terminal domain of RsiW, bound to σ^W , is released from the membrane into the cytoplasm, where Clp proteases degrade the rest of RsiW, freeing active σ^W . Scissors depict specific proteolytic cleavages, and the partial-circle symbols indicate processive degradation. The membrane topology of PrsW is from reference 11. The TMSs of RasP were predicted using TMPred (48), and its predicted membrane topology is based on analogy to *E. coli* RseP (49) and other IMMPs (30).

IPs, based on whether they utilize a metal ion, a serine residue, or a pair of aspartyl or glutamyl residues to catalyze peptide bond hydrolysis. Here, we focused on intramembrane metalloproteases (IMMPs) of bacteria. IMMPs are often called site 2 proteases (S2Ps) because they are related to human S2P and in many cases they cleave a TMS after an initial cleavage outside the membrane by a site 1 protease (3, 4).

IMMPs of bacteria function in envelope stress responses, sporulation, pheromone production, polar morphogenesis, cell division, signal peptide removal from the cell membrane, and virulence of important human pathogens (4). Despite their diverse and important functions, only two IMMPs have been purified and shown to cleave a physiological substrate within its TMS (3). RseP of *Escherichia coli* has been shown to cleave the anti- σ factor RseA *in vitro* (5). The purified RseA was a C-terminally truncated version designed to mimic prior site 1 cleavage by DegS, since site 1 cleavage is normally required for RseA to serve as a substrate of RseP *in vivo* (6, 7). The other IMMP that has been shown to cleave its substrate *in vitro* is *Bacillus subtilis* SpoIVFB, which cleaves Pro- σ^K (8). Here, we report the purification of a third IMMP, RasP, of *B. subtilis* and cleavage of a C-terminally truncated substrate within its TMS.

RasP (formerly YluC) has been proposed to function much like *E. coli* RseP, as an IMMP in a RIP pathway that leads to activation of a σ factor and governs envelope stress responses (9). A model of the RasP-dependent σ^W activation pathway of *B. subtilis* is depicted in Fig. 1. Envelope stressors such as alkaline shock or antimicrobial peptides cause the site 1 protease PrsW (also called YpdC) to cleave the C-terminal extracytoplasmic portion of the anti- σ factor RsiW (10, 11). The remaining C-terminal extracytoplasmic portion of RsiW is susceptible to bulk proteases that trim it to within approximately 13 residues of the TMS (12). Next, RasP is proposed to cleave within the TMS of RsiW, releasing the N-terminal domain of RsiW bound to σ^W into the cytoplasm (9). Finally, the N-terminal domain of RsiW is further degraded by ClpXP and ClpEP (13), allowing σ^W to direct transcription of ~60 to 90 genes whose products defend the cell against membrane-active antimicrobials (14).

Support for the proposed role of RasP in the σ^W activation pathway includes sequence similarity of RasP to IMMPs and analysis of a *B. subtilis* *rasP* null mutant that fails to activate σ^W (9). Complementation of the mutant with *rasP* restored σ^W activation. A *rasP* E21A allele failed to complement, consistent with the prediction that E21 in the HEXXH metalloprotease motif of the putative IMMP would be crucial for proteolytic activity. However, accumulation of RasP E21A was not verified since antibodies were not available. A subsequent study detected a RasP-dependent cleavage product of RsiW that was stabilized somewhat in *clpP* mutant *B. subtilis* (13), but the product was too unstable to allow the cleavage site to be mapped. Heterologous

coexpression of PrsW and RsiW in *E. coli* resulted in C-terminally truncated RsiW that appeared to be trimmed further by Tsp (also called Prc), a periplasmic tail-specific protease of *E. coli*, and this shortened form of RsiW was no longer observed when RasP was also coexpressed (12). These results suggested that RasP cleaved the shortened form of RsiW; however, neither the cleavage product nor RasP was detected, and RasP E21A was not tested.

A second proposed substrate of RasP is the cell division protein FtsL. *B. subtilis* with a *rasP* mutation forms shorter cells and FtsL is more stable, suggesting that FtsL is normally limiting for cell division due to cleavage by RasP (15). Heterologous coexpression of S-tagged RasP and His-tagged FtsL in *E. coli* appeared to result in degradation of FtsL, whereas coexpression of RasP E21A with FtsL allowed it to accumulate, suggesting that RasP directly cleaves FtsL. However, RasP of the expected size was not detected and accumulation of RasP E21A was not tested, weakening the interpretation of this experiment as well as heterologous coexpression experiments reported subsequently (16).

Evidence for additional substrates of RasP has come from experiments in *B. subtilis*. In one study, secreted proteins tagged at their N termini were further shortened in a RasP-dependent fashion after apparent initial cleavage by signal peptidase, suggesting that RasP, like *E. coli* RseP, removes remnant signal peptides from the cell membrane (17). Impressively, ectopic expression of RasP-His₆ but not RasP-His₆ E21A restored further shortening in a *rasP* mutant, and both His-tagged forms of RasP accumulated similarly. The results support direct cleavage of signal peptides by RasP, but an indirect effect of RasP activity could not be excluded, and the cleavage sites were not mapped. Another study implicated the anti- σ RsiV as a substrate of RasP by comparing degradation of N-terminally green fluorescent protein (GFP)-tagged RsiV in wild-type and *rasP* mutant cells (18).

A limitation of RasP proteolysis studies in *B. subtilis* is that indirect effects cannot be ruled out. Heterologous coexpression of RasP and a potential substrate could provide evidence for a direct effect, since other *B. subtilis* proteins would be absent, but proof that RasP functions as an IMMP would require reconstitution *in vitro* of substrate cleavage within a TMS. Here, we report the development of a heterologous coexpression system in *E. coli* that allows detection of tagged RasP and RasP E21A and provides evidence for RasP proteolytic activity on maltose-binding protein (MBP) fusions to TMS-containing parts of FtsL and C-terminally truncated RsiW. We also report purification of RasP-FLAG₂-His₆ and reconstitution *in vitro* of His₆-MBP-RsiW(73–118) cleavage within its TMS, demonstrating that RasP functions as an IMMP.

RESULTS

RasP can inhibit coexpression of other membrane proteins in *E. coli*. To coexpress RasP and potential substrates in *E. coli* (Fig. 2A), we engineered plasmids to express tagged proteins from T7 RNA polymerase promoters. The two plasmids were transformed separately or in combination into *E. coli* strain BL21(DE3), which can be induced to synthesize T7 RNA polymerase. Initially, plasmids designed to express RasP-His₆ and FtsL-His₆ were tested. Upon induction, transformants accumulated FtsL-His₆ abundantly in the absence of RasP-His₆ and much less abundantly in combination with RasP-His₆, although RasP-His₆ (46 kDa) could not be detected even in a long exposure of the immunoblot (Fig. 2B, lanes 1 to 3). We wanted to be able to detect RasP in order to strengthen the interpretation of experiments comparing active and inactive RasP coexpressed with potential substrates. We note that FtsL-His₆ (12 kDa) appeared to migrate primarily as a dimer (Fig. 2B, lane 1). SDS-resistant dimer formation was observed previously when *B. subtilis* FtsL and DivIC were coexpressed highly in *E. coli* (16).

To possibly improve detection of RasP, we engineered a plasmid to express RasP-FLAG₂-His₆. Surprisingly, RasP-FLAG₂-His₆ was detected by anti-His antibodies (Fig. 2B, lane 4), so it was unnecessary to use anti-FLAG antibodies, which are more sensitive. Plasmids engineered to express RasP-FLAG₂-His₆ and FtsL-His₆ were transformed sep-

FIG 2 Effect of RasP on expression of other proteins in *E. coli*. (A) An *E. coli* cell and an expanded view of the inner membrane, showing the predicted topology of RasP and its substrates. The topology of RasP was predicted as explained in the legend to Fig. 1. Here, some additional features of RasP are depicted, including the HELGH motif with its catalytic E21 residue (9), a putative membrane-reentrant β -loop between TMS 1 and TMS 2 (28, 50), a PSD-95/Dgl/ZO-1 (PDZ) domain between TMS 2 and TMS 3 (30), and a short loop interrupting TMS 3 that is predicted on the basis of the structure of a *Methanocaldococcus jannaschii* IMMP (51) and is followed by a conserved DG sequence whose D residue together with the two H residues of the HELGH motif are predicted to coordinate a zinc ion (30). The topology of RsiW was determined in *B. subtilis* (9) and is predicted to be the same in *E. coli*. The topology of FtsL has been predicted previously (52). (B) Effect of RasP-His₆ on accumulation of FtsL-His₆. *E. coli* bearing pYZ20 to express FtsL-His₆ and/or pYZ9 to express RasP-His₆ as indicated at the top was cultured (10 ml) and induced with IPTG. Extracts were subjected to immunoblot analysis with anti-His antibodies, and two exposures are shown. *E. coli* bearing pYZ37 to express RasP-FLAG₂-His₆ was cultured and induced similarly for comparison. (C) Effect of RasP-FLAG₂-His₆ on accumulation of FtsL-His₆ and cytTM-SpoIVFB-FLAG₂-His₆. *E. coli* bearing pYZ20 to express FtsL-His₆, pYZ37 to express RasP-FLAG₂-His₆, pYZ44 to express RasP-FLAG₂-His₆ E21A, and/or pZR209 to express cytTM-SpoIVFB-FLAG₂-His₆, as indicated at the top, was cultured and induced, and extracts were subjected to immunoblot analysis with anti-His antibodies (30-s exposure). In panels B and C, migration of protein markers is indicated at the left, the inferred identity of signals is indicated at the right, and a representative result from at least two biological replicates is shown.

arately or in combination into *E. coli* strain BL21(DE3). Both proteins accumulated separately, and in combination RasP-FLAG₂-His₆ accumulated slightly less abundantly and FtsL-His₆ was barely detectable (Fig. 2C, lanes 1 to 3). To test whether RasP proteolytic activity was responsible for the greatly reduced accumulation of FtsL, we

catalytically inactivated RasP by introducing an E21A substitution as described previously (9). Upon induction, RasP-FLAG₂-His₆ E21A accumulated, but in combination FtsL-His₆ was barely detectable (Fig. 2C, lanes 4 and 5). Therefore, RasP proteolytic activity was not responsible for reduced accumulation of FtsL. Rather, RasP, whether active or inactive, inhibited coexpression of FtsL. An effect of RasP on coexpression of FtsL was noted previously and was attributed to the burden of expressing multiple membrane proteins (16). To test this idea, we examined the effect of RasP on coexpression of *B. subtilis* SpoIVFB, an IMMP (19, 20) with 6 TMSs (21) that is not believed to be a substrate of RasP. We found that both active RasP-FLAG₂-His₆ and the inactive E21A version greatly reduced accumulation of cytTM-SpoIVFB-FLAG₂-His₆ (Fig. 2C, lanes 6 to 8). We conclude that RasP, whether active or inactive proteolytically, can inhibit coexpression of other membrane proteins in *E. coli*. Below, we describe construction of an inactive RasP E21A variant with less of an inhibitory effect on coexpression of other membrane proteins.

Addition of TMSs to RasP allows evidence for proteolytic activity to be obtained in *E. coli*. Addition of cytTM to the N terminus of SpoIVFB enhanced accumulation of cytTM-SpoIVFB-FLAG₂-His₆ expressed in *E. coli* (8). The rationale for adding cytTM, a TMS from rabbit cytochrome P450 2B4 (22), arose from the observation that the N-terminal TMS can affect the recognition and insertion process of the protein translocon (23). We reasoned that RasP might inhibit coexpression of other membrane proteins in *E. coli* by impairing the function of the protein translocon and that adding cytTM might ameliorate this effect. To preserve the usual membrane orientation of cytTM, we also included the first TMS of SpoIVFB, whose C-terminal end is periplasmic when expressed in *E. coli* (21). This end was fused to the N terminus of RasP, which was predicted to be periplasmic in *E. coli* (Fig. 2A). The resulting fusion protein, cytTM-SpoIVFBT_{M1}-RasP-FLAG₂-His₆ (here referred to as 2TM-RasP) (Fig. 3A), and a catalytically inactive derivative, 2TM-RasP E21A, were expressed from T7 RNA polymerase promoters separately or in combination with FtsL-His₆. 2TM-RasP reduced accumulation of FtsL-His₆ much more than did 2TM-RasP E21A (Fig. 3B), suggesting that RasP proteolytic activity was primarily responsible for the reduced accumulation of FtsL-His₆. However, we did not detect a cleavage product (expected to be ~4 kDa smaller) even after a long exposure of the immunoblot. We reasoned that if 2TM-RasP cleaved FtsL-His₆ in its TMS, the C-terminal His₆-tagged fragment might be unstable.

FtsL was His₆ tagged at its N terminus in previous work that suggested it can serve as a substrate for RasP proteolytic activity in *E. coli* (15, 16). Since Clp protease has been reported to degrade IMMP cleavage products released into the cytoplasm of *E. coli* and *B. subtilis* (13, 24), we reasoned that an N-terminal His₆ tag might allow detection of the N-terminal cleavage product of His₆-FtsL in *clpP* mutant *E. coli*. First, we engineered a plasmid to express His₆-FtsL from a T7 RNA polymerase promoter and tested expression alone or in combination with 2TM-RasP or 2TM-RasP E21A in *clpP*⁺ *E. coli*. His₆-FtsL was barely detectable upon coexpression with 2TM-RasP and accumulated more abundantly upon coexpression with 2TM-RasP E21A, although not as abundantly as when expressed alone (Fig. 3C, lanes 1 to 3). This pattern of results suggests that 2TM-RasP is proteolytically active on His₆-FtsL, in agreement with the previous work (15, 16) and strengthening the conclusion since we show that 2TM-RasP E21A accumulates comparably to 2TM-RasP. However, no cleavage product (expected to be ~4 kDa) was detected. Therefore, we constructed a *clpP* mutant derivative of *E. coli* strain BL21(DE3) and repeated the experiment, but still no cleavage product was detected even after a long exposure of the immunoblot (Fig. 3C, lanes 4 to 6).

Having established a system to detect RasP and obtain evidence for proteolytic activity in *E. coli*, we used the system to test for activity on C-terminally truncated RsiW. RasP normally cleaves RsiW after an initial cleavage in its C-terminal extracytoplasmic domain by PrsW (10, 11) and trimming by extracytoplasmic proteases (12) (Fig. 1). Although the exact extent of trimming is unknown, it was estimated to stop at about residue 118 of RsiW (12). Therefore, we engineered a plasmid to express His₆-RsiW(1–118) from a T7 RNA polymerase promoter and tested expression alone or in combina-

FIG 3 Activity of 2TM-RasP on FtsL in *E. coli*. (A) Predicted topology of 2TM-RasP. Two extra TM segments (cytTM and SpoIVFBT1) were added to the N terminus of RasP, creating 2TM-RasP. (B) 2TM-RasP activity on FtsL-His₆. *E. coli* bearing pYZ20 to express FtsL-His₆, pYZ29 to express 2TM-RasP, pYZ53 to express 2TM-RasP E21A, and/or pYZ37 to express RasP-FLAG₂-His₆, as indicated at the top, was cultured (10 ml) and induced with IPTG. Extracts were subjected to immunoblot analysis with anti-His antibodies, and two exposures are shown. Migration of protein markers is indicated at the left, and the inferred identity of signals is indicated at the right. (C) 2TM-RasP activity on His₆-FtsL. Wild-type (+) or *clpP* mutant (–) *E. coli* bearing pYZ66 to express His₆-FtsL alone or in combination with pYZ29 or pYZ53 to express 2TM-RasP or 2TM-RasP E21A, respectively, as indicated at the top, was cultured and induced, and extracts were subjected to immunoblot analysis with anti-His antibodies (3- and 5-min exposures for the left and right blots, respectively). Migration of protein markers is indicated at the left or right, and the inferred identity of signals is indicated in the center. In panels B and C, a representative result from at least two biological replicates is shown.

tion with 2TM-RasP or 2TM-RasP E21A. Expression of His₆-RsiW(1–118) alone resulted in a species migrating at the expected position (12 kDa), and this species was greatly diminished upon coexpression with 2TM-RasP but only slightly more abundant upon coexpression with 2TM-RasP E21A (see Fig. S1A in the supplemental material). Although the small difference in accumulation of His₆-RsiW(1–118) was reproducible in multiple experiments, this pattern of results does not provide convincing evidence for proteolytic activity of 2TM-RasP on His₆-RsiW(1–118).

Our finding that catalytically inactive 2TM-RasP E21A strongly inhibited coexpression of His₆-RsiW(1–118) (Fig. S1A) was reminiscent of the effect of RasP-FLAG₂-His₆ E21A on cytTM-SpoIVFB-FLAG₂-His₆ (Fig. 2C, lanes 6 to 8). Therefore, we examined the effects of 2TM-RasP and 2TM-RasP E21A on coexpression of the putative nonsubstrate cytTM-SpoIVFB-FLAG₂-His₆. Surprisingly, 2TM-RasP greatly reduced accumulation of cytTM-SpoIVFB-FLAG₂-His₆ to a barely detectable level (Fig. S1B, lane 1). 2TM-RasP E21A allowed better accumulation of cytTM-SpoIVFB-FLAG₂-His₆, although not as much as

when expressed alone (Fig. S1B, lanes 2 and 3). This pattern of results suggests that 2TM-RasP is proteolytically active on the putative nonsubstrate cytTM-SpoIVFB-FLAG₂-His₆, raising the possibility that 2TM-RasP is completely promiscuous (i.e., would cleave any coexpressed membrane protein) upon overexpression in *E. coli*.

We examined the effects of 2TM-RasP and 2TM-RasP E21A on coexpression of two other putative nonsubstrates, GFP-Δ27BofA and SpoIVFA. These proteins inhibit SpoIVFB activity during *B. subtilis* sporulation (25, 26). Both proteins are predicted to have a single TMS and a periplasmic C-terminal domain when expressed in *E. coli*, based on analysis of PhoA fusion proteins (21, 27). Hence, the membrane topology of GFP-Δ27BofA and SpoIVFA is expected to be the same as that of FtsL and RsiW (Fig. 2A). Interestingly, coexpression with 2TM-RasP reduced GFP-Δ27BofA accumulation and coexpression with 2TM-RasP E21A had little effect (Fig. S1C), suggesting that 2TM-RasP is proteolytically active on GFP-Δ27BofA. In contrast, neither 2TM-RasP nor 2TM-RasP E21A had much effect on accumulation of SpoIVFA (Fig. S1D), indicating that 2TM-RasP is not completely promiscuous when overexpressed in *E. coli*. We conclude that comparison of the effects of 2TM-RasP and 2TM-RasP E21A on accumulation of a potential substrate upon coexpression in *E. coli* can provide evidence for proteolytic activity, but 2TM-RasP appears to promiscuously cleave some putative nonsubstrates upon heterologous overexpression. Since SpoIVFA has a large extracytoplasmic domain and the extracytoplasmic parts of SpoIVFB and BofA are small, the size of the extracytoplasmic segments may primarily determine whether a membrane protein is cleaved by 2TM-RasP upon coexpression in *E. coli* (see Discussion).

In an effort toward reconstituting RasP cleavage of substrates *in vitro*, we tried to purify His₆-FtsL and His₆-RsiW(1–118), but the yields were very low (data not shown). To improve the yields and perhaps allow better accumulation of substrates upon coexpression with inactive 2TM-RasP E21A in our *E. coli* system, we fused the *E. coli* maltose-binding protein (MBP) to portions of FtsL and RsiW as described in the next section.

Evidence that 2TM-RasP is proteolytically active on His₆-MBP-FtsL and His₆-MBP-RsiW fusion proteins. We constructed two genes designed to fuse His₆-MBP to the 23rd residue of FtsL, which precedes the sequence KKRAS (residues 25 to 29) shown previously to be important for proteolytic activity of RasP on FtsL (15) (Fig. 4A). One gene was designed to fuse His₆-MBP to otherwise full-length FtsL, designated His₆-MBP-FtsL(23–117) (Fig. 4B). The other gene was designed to fuse His₆-MBP to C-terminally truncated FtsL, designated His₆-MBP-FtsL(23–68). The latter was designed to be comparable to RsiW(1–118), with the C terminus 13 to 15 residues beyond the predicted TMS (Fig. 4A). It seemed possible that FtsL, like RsiW, would be subject to C-terminal trimming prior to RasP cleavage *in vivo* (12), so we wanted to produce comparable substrates to eventually test for RasP cleavage *in vitro*. First, though, we tested whether the fusion proteins (expressed from a T7 RNA polymerase promoter) appear to be substrates for 2TM-RasP in *E. coli*. Expression of His₆-MBP-FtsL(23–117) alone in *E. coli* resulted in three species (Fig. 4C, bottom panel, lane 1). The slowest-migrating species had an apparent molecular mass of about 52 kDa, as expected for the full-length protein. Presumably, the two faster-migrating species resulted from proteolysis upon overexpression (Fig. 4C, bottom panel, lane 1, dots). Interestingly, the fastest-migrating species comigrated with a species observed when His₆-MBP-FtsL(23–68) alone was expressed in *E. coli* (Fig. 4C, bottom panel, lane 4, dot). This species appeared to be slightly smaller than full-length His₆-MBP-FtsL(23–68), suggesting that it lacks nearly all of the C-terminal part predicted to be exposed to the periplasm (Fig. 4A). We considered the possibility that *E. coli* RseP was cleaving both His₆-MBP-FtsL proteins in their TMS, but this was not the case since an *rseP* mutant derivative of *E. coli* strain BL21(DE3) expressing His₆-MBP-FtsL(23–117) alone produced the same species (see Fig. S2, lanes 1 and 2, in the supplemental material). Coexpression of 2TM-RasP with either His₆-MBP-FtsL fusion protein greatly diminished the abundance of all species, and coexpression with 2TM-RasP E21A had much less effect (Fig. 4C, bottom panel), suggesting that all species are substrates of 2TM-RasP, although no apparent cleavage products were detected. We note that 2TM-RasP and 2TM-RasP E21A are

FIG 4 Activity of 2TM-RasP on MBP fusions to FtsL and RsiW in *E. coli*. (A) Sequences of FtsL and RsiW aligned at their KKRAS sequences. Gray background indicates identical residues. Predicted TM segments are underlined (15). Numbers indicate residues in FtsL and RsiW that were included in particular fusion proteins. The arrow indicates the cleavage site *in vitro* based on mass spectrometry analysis. (B) Predicted topology of His₆-MBP fusion proteins. Two fusions to FtsL and six fusions to RsiW were created as potential substrates for RasP. (C) 2TM-RasP activity on His₆-MBP-FtsL fusion proteins. *E. coli* bearing pYZ112 or pYZ111 to express His₆-MBP-FtsL(23-117) or His₆-MBP-FtsL(23-68), respectively, alone or in combination with pYZ29 or pYZ53 to express 2TM-RasP or 2TM-RasP E21A, respectively, as indicated at the top, was cultured (10 ml) and induced with IPTG. Extracts were subjected to immunoblot analysis with anti-FLAG (5-min exposure) or anti-His (1.5-min exposure) antibodies in the top and bottom panels, respectively. The dots indicate presumed proteolytic breakdown products independent of RasP activity. (D) 2TM-RasP activity on His₆-MBP-RsiW fusion proteins. The procedure was as for panel C except that *E. coli* bearing pYZ69, pYZ75, pYZ76, or pYZ115 to express His₆-MBP-RsiW(73-118), His₆-MBP-RsiW(80-118), His₆-MBP-RsiW(87-118), or His₆-MBP-RsiW(73-122), respectively, was cultured and induced, and extracts were subjected to immunoblot analysis with anti-His antibodies. Two exposures are shown. (E) 2TM-RasP activity on additional His₆-MBP-RsiW fusion proteins. The procedure was as for panel C except that *E. coli* bearing pDP56 or pDP57 to express His₆-MBP-RsiW(87-168) or His₆-MBP-RsiW(87-208), respectively, was cultured and induced, and extracts were subjected to immunoblot analysis with anti-FLAG (5-min exposure) or anti-His (30-s exposure) antibodies in the top and bottom panels, respectively. The dots indicate abundant presumed proteolytic breakdown products independent of RasP activity. The asterisk

(Continued on next page)

undetectable with anti-His antibodies in the exposure shown in the bottom panel of Fig. 4C and that 2TM-RasP reproducibly accumulated better than 2TM-RasP E21A when each was coexpressed with His₆-MBP-FtsL(23–117), as detected with anti-FLAG antibodies (Fig. 4C, top panel, lanes 2 and 3). However, 2TM-RasP and 2TM-RasP E21A accumulated similarly upon coexpression with His₆-MBP-FtsL(23–117) in *clpP* mutant *E. coli*, although still no cleavage product (expected to be ~7 kDa smaller) was detected (see Fig. S3, lanes 2 and 3, in the supplemental material). Likewise, 2TM-RasP and 2TM-RasP E21A accumulated similarly upon coexpression with His₆-MBP-FtsL(23–68) in *clpP*⁺ *E. coli*, and no cleavage product (expected to be ~2 kDa smaller) was detected (Fig. 4C, lanes 5 and 6). 2TM-RasP E21A reproducibly accumulated better than 2TM-RasP upon coexpression with His₆-MBP-FtsL(23–68) in *clpP* mutant *E. coli*, yet only active 2TM-RasP caused complete loss of His₆-MBP-FtsL(23–68), and still no cleavage product was detected (Fig. S3, lanes 5 and 6).

We also constructed genes designed to fuse His₆-MBP to portions of RsiW. One gene was designed to fuse His₆-MBP to the 73rd residue of C-terminally truncated RsiW, designated His₆-MBP-RsiW(73–118) (Fig. 4A and B). This fusion protein (expressed from a T7 RNA polymerase promoter) was almost undetectable upon coexpression with 2TM-RasP, but it accumulated considerably upon coexpression with 2TM-RasP E21A (Fig. 4D, lanes 2 and 3). The difference in accumulation of His₆-MBP-RsiW(73–118) upon coexpression with active versus inactive 2TM-RasP strongly suggests that His₆-MBP-RsiW(73–118) is a substrate. As for the other apparent substrates, a detectable cleavage product (expected to be ~2 kDa smaller) failed to accumulate even in *clpP* mutant *E. coli* (see Fig. S4 in the supplemental material).

We also tested three variants of His₆-MBP-RsiW(73–118) that had deletions of the sequence KKRAS (leaving residues 80 to 118 of RsiW) or KKRASVKRWFR (leaving residues 87 to 118 of RsiW) preceding the predicted TMS or had four additional residues at the C terminus (QPNL, hence residues 73 to 122 of RsiW) (Fig. 4A and B). The three variants were indistinguishable from His₆-MBP-RsiW(73–118); they were almost undetectable upon coexpression with 2TM-RasP but accumulated considerably upon coexpression with 2TM-RasP E21A (Fig. 4D, lanes 4 to 12), suggesting that they are substrates of 2TM-RasP. Detectable cleavage products failed to accumulate. For His₆-MBP-RsiW(87–118), we varied the growth temperature and the time of sample collection postinduction, but still a detectable cleavage product (expected to be ~2 kDa smaller) failed to accumulate (see Fig. S5 in the supplemental material). We conclude that in *E. coli*, the KKRAS sequence of RsiW is not important for proteolytic activity of RasP. Also, leaving an additional 4 residues on the C-terminal end of RsiW did not interfere with RasP activity.

Having established that His₆-MBP-RsiW(87–118) appeared to be a substrate of 2TM-RasP in our *E. coli* system, we tested two derivatives with different C-terminal ends. His₆-MBP-RsiW(87–208) has a full-length C-terminal end and, as expected, was not cleaved by 2TM-RasP (Fig. 4E, bottom panel, lanes 1 to 3). In contrast, His₆-MBP-RsiW(87–168), which was designed to mimic the C-terminal end after cleavage by PrsW, appeared to be largely cleaved by 2TM-RasP, although some uncleaved His₆-MBP-RsiW(87–168) remained (Fig. 4E, bottom panel, lanes 4 to 6). Two prominent faster-migrating species also appeared to be substrates for 2TM-RasP (Fig. 4E, bottom panel, dots). The faster-migrating species are not due to *E. coli* RseP (Fig. S2, lanes 3 and 4) but may result from trimming by Tsp and/or activity of other periplasmic proteases in *E. coli*, as reported previously for RsiW upon coexpression with PrsW (12). However, in the previous study, His₆-RsiW cleaved by PrsW failed to accumulate, as it was immediately trimmed by Tsp, whereas His₆-MBP-RsiW(87–168) accumulated in our system if 2TM-

FIG 4 Legend (Continued)

indicates 2TM-RasP ± E21A, which is weakly detected by anti-His antibodies in lanes 5 and 6 and comigrates with strongly detected His₆-MBP-RsiW(87–208) in lanes 2 and 3. In panels C to E, migration of protein markers is indicated at the left and the inferred identity of signals is indicated at the right, and a representative result from at least two biological replicates is shown.

RasP was absent or catalytically inactive (Fig. 4E, bottom panel, lanes 4 and 6). We conclude that His₆-MBP-RsiW(87–168), but not His₆-MBP-RsiW(87–208), appears to serve as a substrate for 2TM-RasP in our *E. coli* system.

Taken together, our results with N-terminal His₆-MBP fusions to TMS-containing parts of FtsL and RsiW provide strong evidence that RasP is proteolytically active on the fusion proteins upon heterologous coexpression in *E. coli*, with the notable exception that His₆-MBP-RsiW(87–208) with a full-length C-terminal end resists cleavage.

RasP cleaves His₆-MBP-RsiW(73–118) *in vitro*. 2TM-RasP did not accumulate significantly better than RasP-FLAG₂-His₆ when expressed in *E. coli* (Fig. 3B, right panel, lanes 3 and 6), so we opted to purify RasP-FLAG₂-His₆. We reasoned that the latter, lacking the 2 extra TMSs at the N-terminal end of 2TM-RasP, might be easier to solubilize from membranes and would likely be active. RasP-FLAG₂-His₆ was solubilized from *E. coli* membranes with the nonionic detergent *n*-decyl- β -D-maltoside (DM) and purified by cobalt affinity chromatography followed by gel filtration. As a control, we purified RasP-FLAG₂-His₆ E21A, which was expected to be inactive. Similarly, we purified His₆-MBP-RsiW(73–118), His₆-MBP-FtsL(23–117), and His₆-MBP-FtsL(23–68) as potential substrates. Each potential substrate was incubated with RasP-FLAG₂-His₆, the E21A variant, and alone in separate reaction mixtures containing detergent (0.1% DM) and zinc acetate (6 μ M). Only incubation of RasP-FLAG₂-His₆ with His₆-MBP-RsiW(73–118) resulted in a cleavage product (Fig. 5A, lanes 2 and 3). The cleavage product appeared to be produced by proteolytic activity of RasP-FLAG₂-His₆ rather than a contaminating protease from *E. coli* since it was not observed with the E21A variant or the substrate alone (Fig. 5A, lanes 4 and 1, respectively). RasP-FLAG₂-His₆ did not appear to cleave either of the potential His₆-MBP-FtsL substrates (Fig. 5B). We considered the possibility that a cleavage product comigrated with the fastest-migrating species (marked with an asterisk); however, mass spectrometry analysis of the reactions shown in lanes 4 (substrate alone) and 5 (substrate plus enzyme) of Fig. 5B produced nearly identical patterns in the mass range expected for the faster-migrating species (see Fig. S6 in the supplemental material). The masses of the most abundant species in this range were consistent with the predicted mass of His₆-MBP-FtsL(23–58) alone (44,860.2 Da) and with NaCl (44,918.7 Da). This corresponds to a C terminus several residues beyond the end of the predicted FtsL TMS (Fig. 4A). Hence, the faster-migrating species may result from trimming by Tsp and/or activity of other periplasmic proteases in *E. coli*, but neither this species nor the longer potential His₆-MBP-FtsL substrates appear to be cleaved in their TMS by RasP-FLAG₂-His₆. Only His₆-MBP-RsiW(73–118) appeared to serve as a substrate of RasP-FLAG₂-His₆ *in vitro*.

To follow up on the apparent cleavage of His₆-MBP-RsiW(73–118) by RasP-FLAG₂-His₆ (Fig. 5A, lanes 2 and 3), a sample of the reaction mixture was subjected to mass spectrometry analysis. Strikingly, the most abundant species had a mass of $43,626 \pm 5$ Da (Fig. 6), in excellent agreement with the predicted mass of His₆-MBP-RsiW(73–94) (43,625.6 Da). This result implies that RasP-FLAG₂-His₆ cleaved His₆-MBP-RsiW(73–118) between Ala-94 and Val-95 in the TMS (Fig. 4A). The second most abundant species had a mass of $46,366 \pm 5$ Da (Fig. 6), consistent with the predicted mass of uncleaved His₆-MBP-RsiW(73–118) (46,361.7 Da). The lower abundance of uncleaved substrate than of cleavage product in the mass spectrometry analysis was unexpected based on their apparent abundance from the Coomassie blue-stained gel (Fig. 5A, lanes 2 and 3). The presence of an intact TMS in the uncleaved substrate but not in the cleavage product might explain the discrepancy, since TMSs can impair ionization or other steps in the mass spectrometry analysis. As a control, the reaction mixture in which His₆-MBP-RsiW(73–118) had been incubated alone (Fig. 5A, lane 1) was analyzed by mass spectrometry, and as expected, the uncleaved substrate but not the cleavage product was observed (see Fig. S7 in the supplemental material). As a further control, a reaction mixture in which RasP-FLAG₂-His₆ had been incubated alone was analyzed, and as expected, neither the uncleaved substrate nor the cleavage product was observed (see Fig. S8 in the supplemental material). Neither was RasP-FLAG₂-His₆ (50,067.9 Da)

FIG 5 RasP cleaves His₆-MBP-RsiW(73-118) but not His₆-MBP-FtsL fusion proteins or His₆-MBP-RsiW(87-208) *in vitro*. (A) Cleavage of His₆-MBP-RsiW(73-118) by RasP-FLAG₂-His₆. Purified His₆-MBP-RsiW(73-118) was incubated for 4 h alone or in combination with purified RasP-FLAG₂-His₆ or RasP-FLAG₂-His₆ E21A as indicated at the top. (B) RasP-FLAG₂-His₆ fails to cleave His₆-MBP-FtsL fusion proteins. Purified His₆-MBP-FtsL(23-68) or His₆-MBP-FtsL(23-117) was incubated for 12 h alone or in combination with purified RasP-FLAG₂-His₆ or RasP-FLAG₂-His₆ E21A as indicated at the top. (C) Dependence of His₆-MBP-RsiW(73-118) cleavage by RasP-FLAG₂-His₆ on inhibitors and zinc. 1,10-Phenanthroline (5 mM) in ethanol, ethanol alone, or EDTA (5 mM) was added to the reaction mixture, or zinc acetate was omitted, as indicated. (D) RasP-FLAG₂-His₆ fails to cleave His₆-MBP-RsiW(87-208). Purified His₆-MBP-RsiW(73-118) as a control or His₆-MBP-RsiW(87-208) was incubated with purified RasP-FLAG₂-His₆, or each protein was incubated separately as a control, as indicated at the top. The observed cleavage product from His₆-MBP-RsiW(73-118) (lane 1) is 6 residues longer than that expected if His₆-MBP-RsiW(87-208) were cleaved at the same position, but no cleavage product was observed (lane 3). The line indicates that intervening lanes were omitted from the image. For all panels, samples were subjected to SDS-PAGE followed by Coomassie blue staining. Migration of protein markers is indicated at the left, and purified proteins are indicated at the right. In panels A, C, and D, the location of the apparent cleavage product is indicated at the right. In panel B, the asterisk at the left indicates the faster-migrating species that copurified with both His₆-MBP-FtsL fusion proteins. A representative result from at least two independent *in vitro* reactions is shown.

observed (Fig. S8), presumably because its four TMSs impaired ionization or other steps, although a low-abundance species at $50,069 \pm 5$ Da was detected from the reaction of RasP-FLAG₂-His₆ with His₆-MBP-RsiW(73-118) (Fig. 6).

We conclude that purified RasP-FLAG₂-His₆ cleaved His₆-MBP-RsiW(73-118) within its TMS, demonstrating that RasP is an IMMP. RasP-FLAG₂-His₆ failed to cleave N-terminally His₆-MBP-tagged parts of FtsL beginning with residue 23, spanning the TMS, and ending with residue 117 (the last residue of native FtsL), residue 68 (designed to be comparable to C-terminally trimmed RsiW), or residue 58 (produced by *E. coli* proteolytic activity); possible reasons are discussed below.

FIG 6 Mass spectrometry analysis of the reaction mixture containing His₆-MBP-RsiW(73–118) plus RasP-FLAG₂-His₆. The zero charge state signal as a percentage of the maximum signal is plotted in the mass range from 39,000 to 52,000 Da. Numbers above peaks give the zero charge state mass (in daltons) at peak maxima. The implied cleavage product His₆-MBP-RsiW(73–94) and uncleaved His₆-MBP-RsiW(73–118) are indicated.

Having established that purified RasP-FLAG₂-His₆ cleaved His₆-MBP-RsiW(73–118) within its TMS, we characterized the *in vitro* reaction further. Reducing the reaction temperature to 25°C nearly eliminated cleavage (see Fig. S9A in the supplemental material). The enzyme appeared to be slightly less active at a high salt concentration (Fig. S9B). A time course showed that product accumulates steadily for at least 16 h at 37°C (Fig. S9C), so subsequent reaction mixtures were incubated under these conditions. A titration of the enzyme showed that the amount of product was approximately proportional to the amount of enzyme added to the reaction mixture over the range tested (Fig. S9D), which covered the testable range given the concentrations of the enzyme and substrate that we purified. As expected, the reaction was inhibited by the metal chelator 1,10-phenanthroline (Fig. 5C, lane 2), as observed previously for *E. coli* RseP (5) and *B. subtilis* SpoIVFB (8). Interestingly, the reaction was not inhibited by EDTA (Fig. 5C, lane 4), perhaps because this metal chelator is highly charged and unable to access the RasP active site. Also, the reaction did not require zinc addition (Fig. 5C, lane 7), as did SpoIVFB (8), suggesting that RasP retained its metal ion during purification. Finally, His₆-MBP-RsiW(87–208) was not cleaved (Fig. 5D), consistent with our finding that the full-length C-terminal end blocks cleavage *in vivo* (Fig. 4E).

DISCUSSION

We have established a system for heterologous coexpression of *B. subtilis* RasP with putative substrates in *E. coli*. We have used the system to provide evidence that RasP cleaves FtsL, but putative nonsubstrates with comparably short extracytoplasmic segments also appeared to be cleaved. We found that C-terminally truncated His₆-MBP-RsiW fusion proteins accumulate better than C-terminally truncated His₆-RsiW(1–118) upon coexpression with inactive RasP and provide stronger evidence for proteolysis by active RasP. Moreover, His₆-MBP fusion proteins could be purified, allowing us to demonstrate RasP intramembrane proteolytic activity *in vitro*. To our knowledge, this is only the third instance of an IMMP being purified and shown to cleave a physiological substrate in its TMS. Interestingly, purified RasP cleaved His₆-MBP-RsiW(73–118) but failed to cleave His₆-MBP-FtsL(23–117) or two C-terminally truncated derivatives (ending with residue 68 or residue 58). We conclude that the FtsL fusion proteins have one

or more additional requirements for cleavage, which are unmet *in vitro* but are satisfied upon coexpression in *E. coli*.

Heterologous coexpression of IMMPs and potential substrates. Our results reveal both potential pitfalls and potential solutions in efforts to provide evidence for proteolytic cleavage by IMMPs using heterologous coexpression approaches. First, we found that RasP-His₆ did not accumulate detectably despite overexpression using the powerful T7 RNA polymerase system. In a previous study, overexpression of S-tagged RasP in *E. coli* led to accumulation of a species much smaller than expected, suggesting that RasP was susceptible to degradation (15). We found that addition of FLAG₂ to RasP-His₆ allowed detection of RasP-FLAG₂-His₆ of the expected size (Fig. 2B). We do not understand why FLAG₂ improved accumulation of RasP-FLAG₂-His₆, but we note that the same C-terminal tags permitted accumulation of another IMMP, SpoIVFB (8).

Unfortunately, RasP-FLAG₂-His₆ inhibited coexpression of potential substrate and nonsubstrate membrane proteins irrespective of the catalytically inactivating E21A substitution (Fig. 2C). These results highlight a second potential pitfall in heterologous coexpression experiments. Loss of potential substrate might be misinterpreted as evidence for IMMP-mediated proteolysis unless a control experiment with a catalytically inactive IMMP is performed. The addition of two TMSs to RasP-FLAG₂-His₆ E21A partially or completely relieved inhibition of coexpression of most other membrane proteins. A notable exception was His₆-RsiW(1–118), which prevented us from obtaining convincing evidence for RasP proteolytic activity on this potential substrate (see Fig. S1A in the supplemental material). This problem was overcome by fusing N-terminal His₆-MBP to C-terminally truncated RsiW (Fig. 4D).

A third potential pitfall of heterologous coexpression is that cleavage products may be less stable in the heterologous host than in the native organism. In prior work, neither immunoblot nor pulse-chase immunoprecipitation analysis revealed a RasP cleavage product of RsiW upon coexpression in *E. coli*, suggesting that the putative cleavage product was unstable (12). In agreement, we were unable to detect RasP cleavage products in *E. coli*, including in a *clpP* mutant (Fig. 3C; see Fig. S3 and S4 in the supplemental material). Additional mutations in *E. coli* protease genes might overcome this limitation of our current system.

Our experiments with putative nonsubstrates uncovered promiscuity as a fourth potential pitfall of heterologous coexpression. Since the effect of a *rasP* mutation on stability of SpoIVFB and BofA during *B. subtilis* sporulation has not been reported, we cannot be certain whether the apparent proteolytic activity of 2TM-RasP on cytTM-SpoIVFB-FLAG₂-His₆ (Fig. S1B) and GFP-Δ27BofA (Fig. S1C) is nonphysiological. SpoIVFB appears to have three short (3- to 8-residue) periplasmic loops (21) and BofA appears to have a short (31-residue) C-terminal periplasmic domain (27), based on analysis of PhoA fusion proteins expressed in *E. coli* and based on a homology model of SpoIVFB (28). In contrast, SpoIVFA appears to have a long (173-residue) C-terminal extracytoplasmic domain (21, 29) and resisted cleavage by 2TM-RasP (Fig. S1D). Conceivably, the single PDZ domain of RasP (30) (Fig. 2A) acts as a size exclusion filter, as proposed for *E. coli* RseP (31, 32), which has a pair of circularly permuted PDZ domains in tandem located in a periplasmic loop (33). Recent studies support a model in which the tandem PDZ domains prevent substrate RseA in complex with RseB from entering the RseP active site (31, 32). RseB interacts with a glutamine-rich region of the RseA periplasmic domain, creating a sizable complex (34, 35). Site 1 cleavage by DegS in the RseA periplasmic domain is proposed to reduce its size and release RseB, allowing C-terminally truncated RseA to enter the active site of RseP (32). Since RseA with its intact 98-residue periplasmic domain can enter the active site of RseP if RseB is absent (36), it seems possible that RasP promiscuously allows coexpressed membrane proteins with small extracytoplasmic domains to enter its active site and be cleaved. Promiscuity may be an important feature of RseP and RasP that allows them to indiscriminately remove remnant signal peptides from membranes (17). Perhaps promiscuity also permits IMMPs with PDZ domains to clear excess subunits of membrane proteins that

are normally found in complexes. During *B. subtilis* sporulation, SpoIVFB, BofA, and SpoIVFA form a complex that resists proteolysis, but the absence of any protein from the complex destabilizes the other two proteins (26). Whether the observed instability of SpoIVFB and BofA depends on RasP is unknown. In any case, the results of heterologous coexpression experiments must be interpreted with caution, not only because partner proteins may be missing but because expression levels and other factors may differ from those in the native organism.

Taken together with previous studies, our results reveal strategies that may apply broadly to the development of systems for heterologous coexpression of IMMPS and potential substrates. Addition of N-terminal cytTM (with proper attention to preserve its usual membrane topology, as was done by also adding SpoIVFBT1 in the case of 2TM-RasP) and C-terminal FLAG₂-His₆ proved beneficial for both RasP in this study (Fig. 2 and 3) and for SpoIVFB in a previous study (8). Addition of N-terminal His₆-MBP to potential substrates was beneficial in this study, because such fusion proteins accumulated better than their N-terminally His₆-tagged counterparts upon coexpression with 2TM-RasP E21A and thus provided stronger evidence for proteolysis by 2TM-RasP (Fig. 4). Also, the His₆-MBP fusion proteins could be purified for *in vitro* studies (Fig. 5 and 6). Efforts to develop *E. coli* coexpression systems for other IMMPS and potential substrates, as well as work toward reconstituting proteolysis *in vitro*, might benefit by taking similar approaches.

Evidence that RasP cleaves full-length FtsL and C-terminally truncated RsiW *in vivo*. Part of the motivation for this study was to further investigate whether RasP cleaves full-length FtsL without prior site 1 cleavage. This was suggested by the absence of a detectable site 1 cleavage product in *rasP* mutant *B. subtilis* (15). Given that RseP can cleave full-length RseA with its 98-residue periplasmic domain if RseB is absent (36), it seemed possible that full-length FtsL with its 64-residue extracytoplasmic domain would be cleaved by RasP. Indeed, full-length FtsL-His₆ (Fig. 3B) and His₆-FtsL (Fig. 3C) and His₆-MBP-FtsL(23–117) with an intact extracytoplasmic domain (Fig. 4C) all appeared to be cleaved by 2TM-RasP in *E. coli*. How, then, is FtsL cleavage by RasP regulated in *B. subtilis*? It has been suggested that a RasP recognition sequence, KKRRAS, in the N-terminal cytoplasmic domain of FtsL (Fig. 4A) is shielded from RasP when FtsL interacts with other proteins in the divisome, and it was shown that DivIC interacts with FtsL and stabilizes FtsL against RasP cleavage upon heterologous coexpression in *E. coli* (16). In *B. subtilis*, divisome disassembly after division would uncover the KKRRAS sequence of FtsL, allowing cleavage by RasP. In *E. coli* that is not coexpressing DivIC, the KKRRAS sequence would be exposed, and our results (Fig. 3B and C and 4C) support the notion that RasP can cleave full-length FtsL without prior site 1 cleavage (15, 16).

In contrast to the case for FtsL, there is strong evidence from *in vivo* studies that RsiW undergoes site 1 cleavage by PrsW (10, 11). PrsW appears to remove 40 residues from the 103-residue C-terminal extracytoplasmic domain of RsiW (12). The remaining 63-residue extracytoplasmic portion is similar in length to the 64-residue extracytoplasmic domain of full-length FtsL, yet in a previous study it was concluded that RsiW must be further trimmed by extracytoplasmic proteases in order to be cleaved by RasP (12). The authors engineered *E. coli* to coexpress His₆-RsiW and PrsW. A 14-kDa form of His₆-RsiW accumulated unless an *E. coli* *tsp* mutant was used, in which case a 19-kDa form accumulated. Further experiments provided evidence that the 19-kDa form was the PrsW cleavage product, the 14-kDa form resulted from trimming by Tsp, and only the 14-kDa form could be cleaved by RasP.

Our results support the requirement for site 1 cleavage of RsiW, but not the need for further trimming by extracytoplasmic proteases, prior to cleavage by RasP in our *E. coli* system. The requirement for site 1 was demonstrated by our finding that His₆-MBP-RsiW(87–208) was not cleaved by 2TM-RasP (Fig. 4E). In contrast, His₆-MBP-RsiW(87–168), which was designed to mimic the product of site 1 cleavage by PrsW, appeared to be cleaved by 2TM-RasP without the need for further trimming by extracytoplasmic proteases. In the context of a model in which the PDZ domain acts as a size exclusion filter (31, 32), our results suggest that the RasP PDZ domain excludes the full-length

103-residue extracytoplasmic domain of RsiW but not the 63-residue extracytoplasmic portion after PrsW cleavage.

Perhaps the high level of expression using T7 RNA polymerase in our *E. coli* system accounts for the differences between our results and those of the previous study (12). Our site 1 cleavage mimic, His₆-MBP-RsiW(87–168), accumulated despite the presence of Tsp (Fig. 4E), whereas in the previous study Tsp immediately trimmed the 19-kDa form of His₆-RsiW resulting from site 1 cleavage by PrsW to a 14-kDa form (12). We observed two abundant breakdown products of His₆-MBP-RsiW(87–168) that may result from Tsp trimming (Fig. 4E, lane 4, dots). The larger and smaller breakdown products may result from Tsp trimming that stops prior to reaching the membrane and upon reaching the membrane, respectively. The smaller breakdown product might correspond to the 14-kDa form of His₆-RsiW observed previously (12). In addition to the high level of expression in our *E. coli* system, the addition of MBP likely contributes to stability of His₆-MBP-RsiW(87–168) and its breakdown products. High-level coexpression of 2TM-RasP may account for the apparent cleavage of His₆-MBP-RsiW(87–168) and its breakdown products that we observed (Fig. 4E, lane 5).

It is unlikely that the PDZ domain of RasP must bind to a particular residue at the C terminus of RsiW or FtsL, as had been proposed for the second of the tandem PDZ domains of *E. coli* RseP after site 1 cleavage of RseA by DegS based on *in vitro* results (37). These results did not agree with a subsequent *in vivo* study (31). Moreover, structural analysis of the tandem PDZ domains of *Aquifex aeolicus* RseP revealed tryptophan and proline residues lining the ligand-binding groove, that would likely be unfavorable for binding the C-terminal end of the substrate, and these residues are conserved in the tandem PDZ domains of *E. coli* RseP (32) and in the PDZ domain of RasP (W237 and P288). Also, His₆-MBP fusions to FtsL or RsiW with several different C-terminal ends all appeared to be cleaved by 2TM-RasP in *E. coli* (Fig. 4).

In addition to the apparent difference between RsiW and FtsL in terms of a requirement for site 1 cleavage, another apparent difference relates to the KKRAS sequence. This sequence appears to be important for recognition of FtsL by RasP in both *B. subtilis* (15) and upon heterologous coexpression in *E. coli* (16). In contrast, the KKRAS sequence in RsiW did not appear to be important for cleavage by 2TM-RasP in our *E. coli* system (Fig. 4A, D, and E). It remains to be tested whether the KKRAS sequence would be dispensable for RsiW cleavage by RasP in *B. subtilis*. Also, it remains to be tested whether the KKRAS sequence would be dispensable for His₆-MBP-FtsL cleavage by 2TM-RasP in our *E. coli* system.

RasP cleaves the TMS of RsiW *in vitro* but not that of FtsL. Our results indicate that purified RasP-FLAG₂-His₆ cleaved His₆-MBP-RsiW(73–118) within its TMS between Ala-94 and Val-95 (Fig. 4A, 5A, and 6), demonstrating that RasP is an IMMP. Only two other IMMPs, RseP (5) and SpoIVFB (8), have been shown to cleave a physiological substrate *in vitro*. In both cases, the cleavage site *in vitro* was the same as that *in vivo*. We were unable to map the *in vivo* cleavage site of RsiW due to instability of the cleavage product, even in *clpP* mutant *E. coli* (Fig. 3C, S3, and S4). However, previous mutational analysis of the four consecutive alanine residues preceding the *in vitro* cleavage site (Fig. 4A) suggested that RasP cleaves RsiW somewhere after alanine residues 92 to 94 in *B. subtilis* (13). These observations agree with the cleavage site that we mapped between Ala-94 and Val-95 by mass spectrometry analysis of *in vitro* reaction products (Fig. 4A, 5A, and 6).

Neither full-length His₆-MBP-FtsL(23–117) nor two C-terminally truncated derivatives (ending with residue 68 or residue 58) appeared to be cleaved by RasP-FLAG₂-His₆ *in vitro* (Fig. 5B; see Fig. S6 in the supplemental material). We hypothesize that a lack of α -helix-destabilizing residues in the TMS of FtsL prevents cleavage (Fig. 4A). Helix-destabilizing residues have been shown to be important for cleavage of RseA by RseP *in vivo* and *in vitro* (5). The helix-destabilizing residues in RseA stabilize its interaction with RseP (38). The predicted TMS of RsiW is immediately preceded by a proline residue and contains three consecutive glycine residues (Fig. 4A), which might destabilize the

TMS enough to allow cleavage by RasP *in vitro*. By substituting helix-stabilizing residues such as leucine into the predicted TMS of His₆-MBP-RsiW(73–118) and helix-destabilizing residues into the predicted TMS of His₆-MBP-FtsL(23–117), we plan to test the importance of helix stability in cleavage by RasP-FLAG₂-His₆ *in vitro*.

Since full-length His₆-MBP-FtsL(23–117) and C-terminally truncated derivatives do appear to be cleaved by 2TM-RasP upon coexpression in *E. coli* (Fig. 4C), we favor the idea that the membrane environment overcomes the lack of α -helix-breaking residues in the FtsL TMS to permit cleavage, although we cannot rule out the possibility that one or more additional proteins conserved between *E. coli* and *B. subtilis* are required. Incorporation of SpoIVFB into liposomes enhanced cleavage of Pro- σ^K relative to incorporation of SpoIVFB into detergent micelles (8). This approach was facilitated by the finding that Pro- σ^K in detergent micelles associated stably with liposomes. Unlike the TMS of FtsL, the prosequence of Pro- σ^K associates peripherally with membranes (39, 40). It would likely be necessary to incorporate FtsL and RasP into the same liposome, either by coinorporation or by liposome fusion, in order to test whether a membrane environment would allow cleavage.

Purified RasP-FLAG₂-His₆ cleaved His₆-MBP-RsiW(73–118) slowly (see Fig. S9C in the supplemental material), consistent with the behavior of RseP (5, 33) and SpoIVFB (8) *in vitro*. In addition to these IMMPs, other types of IPs, including rhomboids (41, 42) and γ -secretase (43, 44), exhibit low rates of catalysis. Substrate gating (i.e., substrate access to the active site) rather than substrate affinity appears to be rate limiting in the case of the rhomboid GlpG (41). Whether a similar mechanism explains the kinetic properties of RasP and other IPs remains to be determined.

RasP-FLAG₂-His₆ behaved differently *in vitro* than most soluble metalloproteases in that His₆-MBP-RsiW(73–118) cleavage was not inhibited by the metal chelator EDTA (Fig. 5C, lane 4). It will be interesting to see whether this is a general characteristic of IMMPs. The effect of EDTA on RseP and SpoIVFB activity has not been reported. Addition of zinc acetate was important for SpoIVFB activity *in vitro*, perhaps because this IMMP required the harsh ionic detergent Sarkosyl for solubilization from membranes, thus depleting the enzyme of zinc (8). Omission of zinc has not been reported for RseP, but like RasP, RseP can be solubilized from membranes with a mild detergent (5), so it may retain zinc during purification.

Purified RasP-FLAG₂-His₆ discriminated between potential His₆-MBP-RsiW substrates with different C-terminal ends, cleaving a truncated one ending with residue 118 but not a full-length one ending with residue 208 (Fig. 5D), consistent with our heterologous coexpression results (Fig. 4D and E). RseP likewise discriminated between truncated and full-length His₆-MBP-RseA substrates *in vitro* (33). Discrimination was lost by an RseP variant with substitutions in TMS 2 (A115V) and the first PDZ domain (G214E) that together appear to alter the conformation of the periplasmic region containing the tandem PDZ domains, based on trypsin sensitivity. Hence, biochemical experiments, as well as genetic and structural studies, support the model that the PDZ domains of RseP act as a size exclusion filter for substrate discrimination (31–33). Our results are consistent with the PDZ domain of RasP playing a similar role. The methods we have established for investigation of RasP proteolytic activity in *E. coli* and *in vitro* can be used to further explore the role of the PDZ domain, as well as many other questions that remain to be addressed.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are described in Table S1 in the supplemental material, and primers are listed in Table S2 in the supplemental material. DNA sequencing was used to confirm the desired sequences in cloned PCR products and genes subjected to site-specific mutagenesis (QuikChange kit; Stratagene).

Cotransformation and induction. Two plasmids with different antibiotic resistance genes and different genes fused to the T7 RNA polymerase promoter were cotransformed into *E. coli* BL21(DE3), and gene expression was induced with isopropyl- β -D-thiogalactopyranoside (IPTG) as described previously (45) except that cultures were shaken at 250 rpm. Briefly, transformants were grown in Luria-Bertani (LB) liquid medium containing 50 μ g/ml kanamycin sulfate and 200 μ g/ml ampicillin overnight at 37°C, diluted 50-fold with fresh LB medium containing antibiotics, and grown until the culture reached the

mid-log phase (about 60 Klett units), at which time IPTG (0.5 mM) was added and the culture was induced for 2 h. In one experiment, the growth temperature and induction time were varied. As controls, plasmids were transformed singly into *E. coli* and cultures were induced in parallel experiments. In some experiments, plasmids were transformed into a derivative of *E. coli* BL21(DE3) with a null mutation in *clpP*. This strain, designated EDP55, was constructed by using bacteriophage P1_{vir} to transduce a *clpP* allele with a chloramphenicol resistance insertion (46) from strain MC4100 *clpP::cat* (a gift from Tania Baker) into BL21(DE3). Likewise, strain EZR489 was constructed by using P1_{vir} to transduce an *rseP* allele with a kanamycin resistance insertion and an *rseA* allele with a chloramphenicol resistance insertion from strain KK377 (5) into BL21(DE3).

Immunoblot analysis. An equivalent amount of cells (based on the optical density of the culture at 600 nm) was collected from 0.5 to 1.0 ml of culture by centrifugation (12,000 × *g*), and cell extracts were prepared, mixed with an equal volume of 2× sample buffer (50 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol [DTT], and 0.03% bromophenol blue), and boiled for 3 min as described previously (45). Equal volumes were subjected to SDS-PAGE and immunoblotting as described previously (47). The SeeBlue Plus2 prestained standard (Invitrogen) was used to judge migration of protein species. Antibodies that recognize His₆ (penta-His; Qiagen; catalog number 34460), FLAG (M2 monoclonal antibody-peroxidase conjugate; Sigma; catalog number A8592), or green fluorescent protein (GFP) (47) were used at a 1:10,000 dilution. Antibodies that recognize SpoIVFA (47) were used at a 1:3,000 dilution. A goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Bio-Rad; catalog number 170-6515) was used at a 1:10,000 dilution to detect the anti-GFP and anti-SpoIVFA antibodies. Signals were detected using a ChemiDoc MP imager (Bio-Rad).

Large-scale overexpression of proteins for purification. For RasP-FLAG₂-His₆ with or without the E21A substitution, the appropriate plasmids were transformed into *E. coli* BL21(DE3) and transformants were selected, grown in a fermentor with vigorous aeration and pH control, induced with IPTG, collected, and stored as described previously (8). For potential substrates, the appropriate plasmids were transformed into *E. coli* BL21(DE3) and induction was performed as described above, with cells grown in 4 liters of LB medium in flasks.

Protein purification. For RasP-FLAG₂-His₆ with or without the E21A substitution, cells (10 g) were resuspended in 30 ml of lysis buffer (phosphate-buffered saline [PBS] containing 0.1 mg/ml lysozyme, 1 mg/ml RNase A, 2 mg/ml DNase I, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM 2-mercaptoethanol, and 10% glycerol), incubated at 37°C for 10 min, and passed 3 times through a French pressure cell (SLM Aminco) at 14,000 lb/in² (96 MPa). The cell lysate was centrifuged at low speed (15,000 × *g* for 15 min at 4°C) to sediment cell debris. The supernatant was centrifuged at high speed (150,000 × *g* for 90 min at 4°C) to sediment membrane vesicles. The pellet was dispersed in 30 ml of resuspension buffer (PBS containing 1 mM Pefabloc SC, 5 mM 2-mercaptoethanol, and 10% glycerol) using a motorized Dounce homogenizer. The suspension was treated with *n*-decyl-β-D-maltoside (DM) (1%) (Anatrace, Maumee, OH) by rotating the mixture for 1 h at 4°C in order to solubilize membrane proteins. The mixture was then centrifuged at 150,000 × *g* for 75 min at 4°C to sediment insoluble material. The supernatant was mixed with 1 ml Talon Superflow metal affinity resin (Clontech) that had been equilibrated with buffer (PBS containing 1% DM, 5 mM 2-mercaptoethanol, and 10% glycerol). The mixture was rotated for 1 h at 4°C. The cobalt resin was sedimented by centrifugation at 708 × *g* for 2 min at 4°C. The cobalt resin was washed 3 times with PBS containing 150 mM NaCl, 10% glycerol, 5 ml with 0.5% DM, 5 ml with 0.1% DM, and 3.5 ml with 0.1% DM and 10 mM imidazole by rotating briefly and then sedimenting the resin as described above. To elute the protein, the resin was mixed with 1 ml PBS containing 150 mM NaCl, 10% glycerol, 0.1% DM, and 400 mM imidazole by rotating the mixture for 5 min at room temperature, and then the resin was sedimented as above. The supernatant was applied to a Superdex 200 gel filtration column (1.0 cm by 30 cm) equilibrated with PBS containing 150 mM NaCl, 5% glycerol, and 0.1% DM. The column was eluted with the same buffer at 0.4 ml/min, and 0.5-ml fractions were collected. Fractions were analyzed by SDS-PAGE followed by Coomassie blue staining of proteins and detection of RasP-FLAG₂-His₆ with or without the E21A substitution by immunoblotting, and 3 or 4 fractions containing the highest concentration of the desired protein were concentrated to 0.5 ml using an Amicon Ultra centrifugal device with a 10-kDa cutoff (Millipore). The final concentration of both enzymes was 0.3 mg/ml as determined using the Bio-Rad protein assay with bovine IgG as the standard. Either the enzymes were added immediately to reaction mixtures for *in vitro* proteolysis as described below, or glycerol was added to a final concentration of 10% and the enzymes were stored at −80° in aliquots until use. One freeze/thaw cycle did not detectably change activity.

For potential substrates, the desired proteins were purified as described above with the following modifications: cells (7.5 g) were resuspended in 23 ml of lysis buffer containing 3 tablets of Roche cComplete Mini protease inhibitors instead of 1 mM PMSF; the pellet containing membrane vesicles was dispersed in 15 ml of resuspension buffer containing 1 tablet of Roche cComplete Mini protease inhibitors instead of 1 mM Pefabloc SC; the cobalt resin was washed 2 times with PBS containing 150 mM NaCl, 10% glycerol, 5 ml with 0.5% DM, and 5 ml with 0.1% DM; and the protein was eluted stepwise by mixing the resin with 1 ml of PBS containing 150 mM NaCl, 10% glycerol, 0.1% DM, and 30 to 450 mM imidazole in 30 mM increments. Fractions were analyzed by SDS-PAGE followed by Coomassie blue staining of proteins and detection of the desired protein by immunoblotting. The fraction containing the highest concentration of the desired protein was further purified by gel filtration, fractions were analyzed, and the desired protein was concentrated as described above. The final concentration of each potential substrate was 1 mg/ml as determined using the Bio-Rad protein assay with bovine IgG as the standard. Either the potential substrates were added immediately to reaction mixtures for *in vitro* proteolysis as

described below or glycerol was added to a final concentration of 10%, and the potential substrates were stored at -80° in aliquots until use. One freeze/thaw cycle did not detectably change the result.

Reaction mixtures for *in vitro* proteolysis with purified proteins. Reaction mixtures contained purified RasP-FLAG₂-His₆ (2 μ g) or RasP-FLAG₂-His₆ E21A (3 μ g) and purified His₆-MBP-RsiW(87–208) (2 μ g), His₆-MBP-RsiW(73–118) (4 μ g), His₆-MBP-FtsL(23–117) (6 μ g), or His₆-MBP-FtsL(23–68) (8 μ g) in 30 μ l PBS (pH 7.2), 150 mM NaCl, 10% glycerol, 0.1% DM, and 6 μ M zinc acetate, unless indicated otherwise. Reaction mixtures were incubated at 37°C for 16 h unless indicated otherwise, and then reactions were terminated by the addition of an equal volume of 2 $\times$ sample buffer and boiling for 3 min before SDS-PAGE followed by Coomassie blue staining of proteins.

Mass spectrometry analysis. Samples of reaction mixtures for *in vitro* proteolysis were submitted to the Mass Spectrometry and Metabolomics Core of the Research Technology Support Facility at Michigan State University. The samples were subjected to desalting on a BetaBasic CN column (10 mm by 1.0 mm) (5- μ m particles; Thermo). Protein was eluted from the column by a gradient using 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B), starting at A/B = 99/1, increasing to A/B = 30/70 at 5 min after injection, and holding at this composition for an additional 5 min. Analysis employed electrospray ionization (positive polarity) and a Xevo G2-S QToF mass spectrometer (Waters). Electrospray ionization mass spectra were deconvoluted to a zero charge state spectrum using the Waters MaxEnt1 software tool.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00381-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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