Supporting Information

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SI Materials and Methods

DNAS

To make plasmid pJH110 (P_{trc} -espP $\Delta 5$), a DNA fragment encoding EspP residues 998–1300 was amplified by PCR using the oligonucleotides 5'-CACTGACAGGCTATA-

 Szabady RL, Peterson JH, Skillman KM, Bernstein HD (2005) An unusual signal peptide facilitates late steps in the biogenesis of a bacterial autotransporter. Proc Natl Acad Sci USA 102(1):221–226. ACACGGCCGCAAACA-3' and 5'-AAAGCCGGCATTACT-GCAAGCTTTAGTC-3' and pJH62 (1) as a template. The resulting PCR product was digested with Eag I and Hind III and cloned into the cognate sites of pHL36 (2).

2. Lee HC, Bernstein HD (2002) Trigger factor retards protein export in *Escherichia coli*. J Biol Chem 277(45):43527-43535.

	1042	1066	1081	1108
	10 20 30	40 50	60 70 80	90 100 110
EspP 1035	5 INGEAGAWARIMSGTGSASGGFSdny	thVQVGVDKKHel	dG-LDLFTGFTVTHTDSSA	.* * * .
gi 81637358 417 gi 401120 670	7 LQQQSAQSVDTLAAYQKQKGGHS TPLOTTVWAEHLNOKOSassk-htdVKHHOS	LHVGAKVQF	N-PQWQLALVASQQKQDV	QQGLININTQNRVLSTALRYD
gi 75406716 354	SGSQHSIWANVHASDRTdp	tTQIGLDVAG	SSSHTGAYLSHQNQDYv	LDDTLSSDVKTIGMGLYHRHD
gi 122065199 115	DQAQSALWTNIAQDKRRydsdAFRAYQqkt	nlRQIGVQKAL	DNGRIGAVFSHSRSDNt	fDEQVKNHATLTMMSGFAQYQ
gi 81320972 1192 gi 1170514 1290	<pre>2 EDRRNAVWTSGIRDTKHyrsqDFRAYRqqt) NEGQYNVWVSNTSMNKNysssQYRRFSsks</pre>	tqTQLGWDQTI	S-NNVQLGGVFTYVRNSN	fDDGIGNSARLAHGAVFGQYG NFDKATSKNTLAQVNFYSKYY
gi 124244 1281 gi 75445158 633	DAEKNSVWMSNTGYGRDyasaQYRRFSskr YRTLGGVWVEYVNSDMHghggDTNNYRaks	tqTQIGIDRSL	S-ENMQIGGVLTYSDSQH	TFDQAGGKNTFVQANLYGKYY OYGPLSGKDTLTKITAYAKON
gi 81857545 1179	DPQQQNIWLETGTQQTDyhsgTHRPYQqtt	nyAHIGIQTGI	T-DRLSVGTILTDERTNNr	fDEGVSARNRSNGAHLFVKGE
gi 75390661 1015	QASSSNSFAALSNGRFRyktg-shiDINGFS	LIVGVSKEI	ESFTYGIFMEAGNANYnsyndfa	pnsipntNSIKGSGDTNYFGIGVLLNTK
gi 81485260 1243 gi 81483352 942	3 KKGQTTVSGETFNIRNKyyen-nkvVSNGAS 2 LELKNNFWLLVSKENLKnkd	SVIKLNQNTKYagyc	htfN-TGISLGFFAGQSTGRY	KTSNENIKSEVLVLGVNAEYK
gi 75383783 2673 gi 75383587 188	3 TRNVDKFSVIYTGGEHKdstlGVSGYEyks 3 SKDSNKVKVFGTRGEYKtdtagVIDYKNyay	tgVLYLNDREAf	T-YGGKYGWSAGIVGSNFefkgd K-LGRGIGWYAGMVHNTFrfkd	tnkgsKERVVSGKLGLHYQAPLNKDD ignsKEROLOGKVGLFKSVPFDDNN
gi 75399818 3411 gi 75383787 1723	SKDSNKVKTFGMKGEYKtdtagVLDYKNnay	gvAYVHENEDI	K-LGKGTGWYTGIVHNTFkfkd	
gi 75383592 1669	SKDSNKMKIFGTKGEYKtdtagVVDYKNeay	gmAYVHENEDI	K-LGKGIGWYTGIVDNTFk	fKDIGKSKEEQIQAKVGLLKS
gi 75539764 630	5 LEQERGLWGAAITDFLQrkktsTSKKYRhvg	VGYAVGASVHM	P-TEDLFSLAFCQFFNNDk	DFVVSKNRTHVYAGSLFFEHF
gi 81440059 114 gi 54040656 738	PDLIASQWAQFFPTSARGRALPPaqlea NGENNSVRLSIQGGHLGhdnngGIARGAtpess	psqqLMIGPDLFVret- -gsygfVRLEGDLMRte	aaG-DVHRAGIFVGHNNLQSsfrga vaG-MSVTAGVYGAAGHSSVdvk	rpllgDKQRKAVNLSGESLGVYWSMT dddGSRAGTVRDDAGSLGGYLNLV
gi 75446523 810 gi 81656715 853	EGFNQDSWGRIRGQHNNFDAGRfsyds	niwfMQLGHDVYQakn-	aaG-TQVTGGMMITLGKQNSdtrdrar	-ainpdlSIDTGKIKTEAYGFGGYYTLM
gi 81633541 1130	CAEDNTVWARIDGTRAHvepeesgaRVSEYDsrs	wllQAGIDALLhs	-sgK-GELIGGLTAHYGGINTdv	ssfygdgsidtrgyglgatltwy
gi 81751910 1312	PSGNNGIWARIEAAHAEIdpKqSTSKASydad- VTSDNGIWARIGGDYSKlqss-rslTNMSQNirt	vilQSGVDGKFye	adT-GKLIGGINALYGSAISri	nsPSGDGDATTSAWGLGGTLTWY
gi 81751650 833 gi 75446488 763	MVDAHGIWARIQGAhdrlePTTLTGmkqei FDNNOWAWGRIEGSHOVtdparSTSGSOreid-	ntfiLQAGVDGQFye vwkLOTGIDVPLvgs-	deN-GKLIAGITGQYGTARSni	vaRDGDGRISTNAWSLGATATWI hsFFGDGRINSAGYGLGTSLTWY
gi 81431569 621	DVGRNGVWSRVEGSASQldpsaSTTGERqdvd-	swkVQFGVDRILagg-	-qeG-SRLVGGLALQYGKADTrv	ssiygngtvdttaygltptltwy
gi 81538852 481	ESADSCVFITPYAGEYRrdpVTSGYSgqr	ygVLLGQNQHF	GDFQLGWHGGYESASTdf	ngTSVGRKEEINTLMLGVQGGMK
gi 81653745 765 gi 81793944 3687	VAYDYTMWAKALGGRITsssd-saqNIASSHthv RVGDTTVWIAGRSRPNRidgdsTANSVRred	ygTQIGIDKSD dgVMAGAERRI	NDSVYGASFASLQSRFk G-ERSLIGVAVGNQDLESw	tNDGQLSSTVKSQVVGVYGSRC sRANDDRADIDATHVGVYAQAD
gi 81802247 2951	LVGDTTVWITGRSLPNRvdgdaNAVSVRred	ngVMAGAERRF	G-ERSVVGIAVGNQDIESw	SREWGDRADVDGTHAGVYGWAD
gi 81732582 492	2 GFKDAGVWVQSLYSDATqdrrdGIAGYNays	ngIAVGADGKV	N-ENLTLGLAYSFINTDVn	GKSGNKTNVDSHAFTLYGGFE
gi 75393819 449 gi 75385611 1534	AATPWNFWAQTLYAHSRqssgtYTPGYQtng TSINRGVWISGLYGINKqriwkNIPKYQnrt	ygINVGVDRRF	N-DESLFGVSLGYQNANIn	iHSYGNEKDVDSYELMAYTGWF yNKKLGKTTVNGHLLSIYSLKE
gi 81554922 65	TNVNRGIWISGLYGVNKqgvwkNIPKYQgrt	tgLTIGADAEF	InNHDVIGIAYSNLESKIk	YNKKLGKTAVHGHLLSVYGLKE
gi 81554936 64	SNIKRGLWMRGMYGTNNhgrvdNMTGYRgin	kgATVGFDAEI	NNNIIGIAYSNVHSVFkf	kngkNNDKELIGSKVISIYGQKE
gi 81554839 2059	 DNVAYGIWAKPFYTDAHqskkgGLAGYKakt DNIVTGIWGMSFYGKIKqnsknSASGYQsnt 	tgIVIGLDTLA	N-NNLMIGAAIGITKTDIkh	qdYKKGDKTDVNGFSFSLYGAQQ knDKNGDRTKAKSNIYSIYGLYN
gi 112710 1963 gi 81793942 669	MDAKFGAWISPFVGNATqkmcnSISGYKsdt	tgGTIGFDGFV	S-DDLALGLAYTRADTDIkl	knNKTGDKNKVESNIYSLYGLYN
gi 14195037 1245	FDYSTNVWGFAFGGFRTIsae-nlvAIDGYKgay-	ggASAGVDIQL	M-EDFVLGVSGAAFLGKMd	sQKFDAEVSRKGVVGSVYTGFL
gi 75539461 1255	LDFSTNIWGSGMGTFSNcatiaGVDGFThra	ggYALGLDTQL	I-EDFLIGGSFAQFFGYTd	sQSFSSRSDQSGYLGTGYVGIF
gi 14195023 1329 gi 75389231 579	LDFSTNVWGSGLGVvedcqnigEFDGFKhhl GAYLFGTWGSAVSNLFYvhds-sgkPIDNWHhrs	tgYALGLDTQL	V-EDFLIGGCFSQFFGKTe	SQSYKAKNDVKSYMGAAYAGIL
gi 14195030 692	2 DVPGKQLSITGITNFFHanhtgDARSYRhmg	ggYLINTYTRI	T-PDAALSLGFGQLFTKSk	DYLVGHGHSNVYFATVYSNIT
gi 75539768 732	FGYGKGLWVAGISNIFHhdrnsVSHGFRris	ggYVIGANSQT	V-TDSVFGVAFSQIFAKSk	DYVVSSAKSQAIAGSAYLSVK
gi 14195071 621 gi 14195006 628	LQTDRGLWIDGIGNFFHvsaseDNIRYRhns MEHKQGFWVSSMTNFLHktgdeNRKGFRhts	ggYVLSVNNEI	T-PKHYTSMAFSQLFSRDk	DYAVSNNEYRMYLGSYLYQYT DCFIAHNNSRTYGGTLFFKHS
gi 75533908 479 gi 75344862 631	LQGDRAFWCAGLSNFFHkdstkTRRGFRhls	ggYVIGGNLHT	C-SDKILSAAFCQLFGRDr	DYFVAKNQGTVYGGTLYYQHN
gi 75539569 541	ADYQRGLWASGLANFLQksgteTKRKFRhhs	agYVLGAYAKT	L-SDDVFSAAFCQLFGRDk	DYLVSKNNSNIYAGSIYYQHT
gi 75539144 533 gi 75539094 548	ADYHRGFWVSGLANFLHKSgsdTKRKFRhns ADYHRGFWVSGLGNFLHksgsdTKRKFRhns	agYALGVYAKT	P-SEDVFSAAFCQLFGKDk	DYLVSKNNANIYAGSLYYQHI DYLVSKNSSTVYAGSIYYQHI
gi 75386488 67 gi 75386487 155	LSSSTNLWVSGIADFLHedqkgNQRSYRhss LCOERGVWGAGIANFLHrdkiNEHGYRhsg	agYALGGGFFT	A-SENFFNFAFCQLFGYDk	DHLVAKNHTHVYAGAMSYRHL DYVVSKNHGTSYSGVVFLEDT
gi 14195016 630	LCSDRGFWAAGVANFLDkdkkgEKRKYRhks	ggYAIGGAAQT	C-SENLISFAFCQLFGSDk	DFLVAKNHTDTYAGAFYIQHI
gi 14195066 631	IYQQRGLWASGTANFFHkdksgTNQAFRhks	ygYIVGGSAED	F-SENIFSVAFCQLFGKDk	DLFIVENTSHNYLASLYLQHR
gi 14195069 637 gi 14195068 538	7 EPFERELWLSGIANFFYrdsmpTRHGFRhis 3 APYEKRFWVAGISNVLHrsgreNQRKFRhvs	ggYALGITATT	P-AEDQLTFAFCQLFARDr	DYFMNTNFAKTYAGSLRLQHD
gi 75539765 543 gi 75539767 56	QSPGNGIWISSLTNSFRkgsteNNHGFRhks	sgYVAGGKFQT	L-QDDIFSVGICQLFGRSk	DFGSAKSKDKAFSGSLYAHHS
gi 75539766 544	SLCSEGFWAAGIKNYLYSNSpaENYVFQhhn	agYAIGMNKHT	L-SENVFSAAFSQLFGKDr	DHANGHVDHQTLSGSFYAHHV
gi 75344861 356 gi 14195070 982	<pre>EDFHRGLWISGISNFFHkdstkVQEGFRhis 2 APSHPGIWIGGIGNAFHqdkqkENAGFRlis</pre>	sgYVVGVSTQP rgYIVGGSMTT	I-SNKVMDLAFCQMLGKSk	DYRLADARSHVYAASIHTKCE DYVVSDIKSQVYAGSLCAQSS
gi 14195047 687 gi 75389174 703	VSRDVGFIASVQALGDYvlny-kqgNRDGFLary RDVGFVASLHALGDYILnvvgdDRDGFLary	ggFQAVAASHY	E-NGGIFGVAFGQLYGQTksr	lydSKDAGNITILSCFGRSYIDVK
gi 14195029 690	DHPGLSITAKALGAYVEhtprqGHEGFSgry	ggYQAALSMNY	T-DHTTLGLSFGQLYGKTn	aNPYDSRCSEQMYLLSFFGQFP
gi 75393101 659	<pre>DHPFWGITGGGLGMMVYqdpreNHPGFHmrs</pre>	sgYSAGMIAGQ	THTFSLKFSQTYTKLn	eRYAKNNVSSKNYSCQGEMLFS
gi 75539771 97 gi 75539775 666	NSFYFDIEGGALGLCLYqknmaEKAGFHmdg NPDHFELSSQGVLLGVRqhnrsGIRGFRmes	agYYTELSVGS	P-SFYKLGFKFASQKTNAk	aNIGHDEVASDYLSFGSYWEVH eHTTSNKVSSKNFFGSVQLRLP
gi 81790068 114	KEHDLEASLQGLGLLINghnreGRKGFRnht	tgYAATTSAKT	A-ARHSFSLGFAQMFSKTr	eRQSPSTTSSHNYFAGLRFDSL
gi 14195026 669	GQIAPTASGEATRLFVHqnsnnDAKGFHmea	tgYSLGTTSNT		eSHSDNSVASHTTTVALQINNP
gi 14195027 676 gi 75539772 651	ERPFLEIQGIADGLFVHqnsipGAPGFRiqs INDSTSFYLEGNGLASHtrqr-nqnEILGFSsrs	tgYSLQASSET	S-LHQKISLGFAQFFTRTk	eIGSSNNVSAHNTVSSLYVELP eHTSNNKVSSHSYLLGATLOTP
gi 75539774 664	PPPRTSLGVAGRGSVAYvlqk-trnAKPGFElfs-	rgYSTQASRST	E-TNHHFALSFSQFYSEIk	eAKSQDKVSANTYFAGAQIHIP
gi 7404420 758	LPDNSWFALQGAATTLFtkqq-krlSYHGYSsas	kgYTVSSQASG	A-HGHKFLLSFSQSSDKMk	eKETNNRLSSRYYLSALCFEHP
gi 14195035 1462 gi 14195036 1478	EVSYNNLWISGLGTMLSqvgtpTSEEFTyys	rgTSVAIDAKP	A-HDVIVGAAFSKMIGKTksl	krmhnyfhkGSEYSYQASVYGGKF kreNNYTHKGSEYSYQASVYGGKP
gi 75539817 1504 gi 14195024 1439	DAAYNNLWLCGIGSFLQkdegeEARSFSyhs DPAFNNFWASAIGSFLRkevsrNSDSFTvhg	rgYSLAIDAKP	R-PEFILGASFSQVFGHAkse	ktaGQYKHKGSDHSFQGTLYAGRS
gi 14195045 704	PTSYLGLLIGGIGAEMRtysaEKESFIsrs	gtTGTTIIRLT	PTLTLSGGATHMFGDSf	VTKLPEFIASEGMVQNVGLTQI
gi 14195025 673	PLQHLCVFGGPVYQIMEqnpkqSSNNLLvqh	agHNVGARIPF	S-FNTILSAALTQLFSSSs	
gi 75539816 661 gi 75383596 725	PIKYACIFGGPISSVMEqngkSYNNFSttq SNKDSEVWFSVLGSGGKlrrdGYASADtrv	VgQNVGIKLPF	S-PNTVVCATFTQLHGSIs	qDNIPGKSNSHMLLGTVAAFKN fNRYAGESKSNMVGVSFYAKOD
gi 81423439 346	PTLASCAWASATVANCK1t COGECECKt ct	laGOVGADTMA	A-DDVTVGAAMNYSKANV+	fNRYGGNSOAKGVGVSLVSRLN

Fig. S1. Alignment of autotransporter β domain sequences. An alignment of 100 autotransporter β domain sequences was obtained by querying the Conserved Domain Database (www.ncbi.nlm.nih.gov/sites/entrez?db=cdd) with the accession no. pfam03797. The structure of the EspP β domain (accession no. 2QQM) was used as a reference to perform the alignment. The parameters were set to display the most diverse members of the pfam03797 family. The most highly conserved residues are shaded red, and less highly conserved residues are shaded blue. Uppercase residues that are unshaded were aligned but not conserved whereas lowercase residues could not be aligned. Only EspP residues 1035–1112 (*Top*) and the corresponding residues in other β domains are shown. EspP residues W1042, G1066, G1081, and Y1108 are denoted with an arrow. EspP residues Y1125, Y1144, P1170, Q1171, W1185, and G1207 were also identified as highly conserved.

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Fig. 52. Effect of the G1081D mutation on the proteolytic maturation of EspP Δ 5. (*A*) AD202 transformed with pJH110 (P_{trc} -espP Δ 5) or a pJH110 derivative encoding EspP Δ 5(G1081D) were subjected to pulse-chase labeling after the addition of 10 μ M isopropyl- β -b-thiogalactopyranoside (IPTG). Immunoprecipitations were performed using the C-terminal anti-EspP antiserum. (*B*) The percent of the passenger domain fragment that was cleaved at each time point in *A* is shown. A comparison of these results with those shown in Fig. 3 reveals that EspP Δ 5(G1081D) undergoes proteolytic processing faster than EspP(G1081D).



Fig. S3. The G1081D mutation does not significantly affect early steps in β domain folding. Plasmids pTEV2 and pTEV4 (1) encode a truncated version of EspP that contains a noncleavable 116-residue passenger domain fragment and a tobacco etch virus (TEV) protease recognition site at residue 974 [EspP* $\Delta 1$ (TEV2)] or 1010 [EspP* $\Delta 1$ (TEV4)]. Previous work has shown that EspP* $\Delta 1$ (TEV2) and EspP* $\Delta 1$ (TEV2/G1066A) are cleaved by TEV protease but that EspP* $\Delta 1$ (TEV4) and EspP* $\Delta 1$ (TEV4/G1066A) rapidly lose their susceptibility to protease digestion because of the apparent incorporation of the passenger domain- β domain junction into a partially folded β barrel structure (2). In this experiment, AD202 were transformed with a derivative of pTEV2 or pTEV4 encoding EspP* $\Delta 1$ (TEV2/G1081D) or EspP* $\Delta 1$ (TEV4/G1081D). Following the addition of 10 μ M IPTG, cells were subjected to pulse-chase labeling and processed as previously described (2). One-third of the cells were untreated, one-third were treated with proteinase K (PK), and one-third were permeabilized and treated with TEV protease. Immunoprecipitations were then conducted using the anti-EspP C-terminal antiserum and proteins were resolved by SDS/PAGE. An unidentified background band (•) appeared in all samples. The observation that EspP* $\Delta 1$ (TEV2/G1081D) is digested by TEV protease but that EspP* $\Delta 1$ (TEV4/G1081D) is largely protected from digestion strongly suggests that the mutation does not interfere significantly with the overall folding of the β domain.

Dautin N, Barnard TJ, Anderson DE, Bernstein HD (2007) Cleavage of a bacterial autotransporter by an evolutionarily convergent autocatalytic mechanism. *EMBO J* 26(7):1942–1952.
 Ieva R, Skillman KM, Bernstein HD (2008) Incorporation of a polypeptide segment into the β-domain pore during the assembly of a bacterial autotransporter. *Mol Microbiol* 67(1): 188–201.



Fig. S4. Immunoprecipitation of EspP-containing polypeptides from nonirradiated samples. Samples from the experiments shown in Fig. 4A (A) and Fig. 4B (B) that were neither UV-irradiated nor treated with PK are shown.



Fig. S5. An ~155-kDa crosslinking product is not observed in a Δskp strain. AD202 and HDB131 (AD202 Δskp) (1) were transformed with pDULEBpa and pRI22 (P_{*lac*}-espP) harboring an amber mutation at residue 1113 (A) or 1214 (B). After the addition of IPTG cells were pulse labeled and UV-irradiated. Immunoprecipitations were subsequently conducted using an anti-Skp antiserum.

1. leva R, Bernstein HD (2009) Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. Proc Natl Acad Sci USA 106(45):19120–19125.



Fig. S6. The G1066A mutation does not affect the release of the EspP β domain from Skp. The experiment shown in Fig. 4A was repeated, except that all samples were UV-irradiated and processed without PK treatment. The results provide additional evidence that EspP(G1066A) dissociates from Skp (and then binds to the Bam complex) at the same rate as wild-type EspP.



Fig. 57. The binding of the wild-type EspP β domain to the Bam complex can be uncoupled from the initiation of passenger domain translocation at low temperature. AD202 were transformed with pDULEBpa and pRI22 (P_{lac} -espP) harboring an amber mutation at residue 1113 (A) or 1214 (B). After the addition of IPTG cultures were shifted to 25 °C and subjected to pulse-chase labeling. Cells were UV-irradiated and PK was added to half of each sample. Immunoprecipitations were subsequently conducted using the indicated antisera.



Fig. S8. A long segment of the EspP passenger domain is in proximity to BamA when translocation stalls. Previous work has shown that the translocation of the passenger domain of an EspP derivative containing a linker insertion at residue 586 [EspP(586TEV)] stalls transiently near the site of the insertion (1). In this experiment AD202 were transformed with pDULEBpa and pRI23 [P_{lac} -espP(586TEV)] harboring an amber codon at the indicated position. Cells were pulse labeled and subjected to a 1-min chase after the addition of 200 μ M IPTG. Half of each sample was UV-irradiated, and equal portions were used for immunoprecipitations with antisera raised against an EspP N-terminal peptide (2), BamA, BamB, or SurA. Although an EspP-BamA crosslinking product was observed when Bpa was introduced into EspP within ~80 residues of the stall point (residue ~600), an EspP-SurA crosslinking product was observed only when the amino acid analog was introduced \geq 80 residues from the stall point. The crosslinking of residue 575 to both BamA and BamB has been previously reported (3).

- 1. leva R, Bernstein HD (2009) Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. Proc Natl Acad Sci USA 106(45):19120–19125.
- 2. Szabady RL, Peterson JH, Skillman KM, Bernstein HD (2005) An unusual signal peptide facilitates late steps in the biogenesis of a bacterial autotransporter. Proc Natl Acad Sci USA 102(1):221–226.
- 3. leva R, Tian P, Peterson JH, Bernstein HD (2011) Sequential and spatially restricted interactions of assembly factors with an autotransporter β domain. Proc Natl Acad Sci USA 108(31): E383–E391.



Fig. S9. The G1081D mutation impairs the membrane integration of the EspP β domain. AD202 transformed with a pJH61 derivative encoding EspP β' (G1081D) were subjected to pulse-chase labeling after the addition of IPTG. Cells were fractionated and equivalent amounts of each fraction were used for immunoprecipitations with an anti-EspP C-terminal antiserum. Although it is possible that the partial loss of EspP β' (G1081D) that occurred during the fractionation procedure resulted from proteolysis, essentially identical results were obtained when a mixture of protease inhibitors (1 mM AEBSF, 800 nM aprotinin, 50 μ M bestatin, 15 μ M E64, 20 μ M leupeptin, 10 μ M pepstatin A) was added to the cell extract.