Supplementary Material for
A peptide signal for adapter protein mediated degradation by the AAA+ protease ClpCP

Peter Prepiak[1] and David Dubnau[1*]

Public Health Research Institute
225 Warren Street
Newark, NJ 07103

[1]Public Health Research Institute, Newark, NJ 07103, USA

[*]Corresponding author: Email, dubnau@phri; Phone, 973-854-3400; Fax, 973-854-3401

Strain and plasmid construction

To prepare a Campbell-like integration vector for alanine scanning mutagenesis, we used plasmid pUS19 (Benson and Haldenwang, 1993).. Primers CCOMKEH (5**'-**GGGG AAG CTT CGG ACA AGG CAC GCC GCC) and NCOMKSB (5'-GGGGATCC ATG AGT CAG AAA ACA GAC GC) were used to amplify the *comk* gene. The resulting PCR fragment was cleaved with *Hind*III and *BamH*I (restriction sites are underlined) and ligated into pUS19. The resulting plasmid was cleaved with *Sal*I and *BamH*I, blunt ended with Klenow polymerase and self ligated, in order to remove first 150 nucleotides from 5' end of the gene. The product, pUS19ComK, was then used as a template for site directed mutagenesis. Individual plasmids, carying mutations, were transformed into *B. subtilis* strain BD1959 (*amyE::comG-lacZ*, in-frame fusion) Mutations were verified by sequence analysis.

To create a system for the expression of *comK* under the control of the xylose-induced *pxyl* promoter, we utilized the construct *PxylA-comK* (Hahn et al., 1996). Plasmids carrying individual mutations were transformed into *B. subtilis* strain BD1826 (*comG-lacZ*, transcriptional fusion). To disrupt the expression of *comK* at the native locus, the resulting strains were transformed with chromosomal DNA from BD2259, carrying a mini*Tn*10 insertion in the promoter of *comK* (Luttinger et al., 1996).

PxylA-comK was also used to construct the deletions ComKΔ(174-192) and ComKΔ(169-192). The plasmids carrying mutations as well as *PxylA-comK* were transformed into *B. subtilis* strain BD1826. Chromosomal DNA purified from the resulting strains was used to transform BD1826 by congression together with chromosomal DNA from strain BD4263 (*comk*::spc). The resulting *B. subtilis* strains, BD4310(wild type), BD4311 (Δ169-192) and BD4312 (Δ174-192) were then transduced (using phage SPP1) with DNA from BD2091 (Δ*mecA*::erm) yielding strains BD4313 (*comK::spc amyE::comK* (wild type) *comG-lacZ*), BD4314 (*comk*::*spc amyE::comKΔ174-192 comG-lacZ*) and BD4315 (*comk*::*spc amyE::comKΔ168-192 comG-lacZ*). Strain BD4263 was created by transforming IS75 with chromosomal DNA from BV2004 (Hamoen et al., 2002).

To demonstrate targeted degradation, we appended the *comK* sequence at the 3' terminus of the coding sequence for GFP. GFP fusions were constructed using the integration vector pSG1729 (Lewis and Marston, 1999). A fusion of *gfp*-to the wild-type *comK* was constructed using primers CKGFPBAM (5'-GGC GG ATC CAC ATG AGT CAG AAA ACA GAC) and CKGFPHIND (5'-CG AGG AAG CTT CTA ATA CCG TTC CCC GAG CTC) and ligating the resulting PCR fragment into *BamH*I and *Hind*III (restriction sites are underlined) sites of pSG1729. A fusion of gfp to the C-terminal part of *comK* (pSG1729-GFP-C) was constructed using primers CKCTGFPBAM (5'-GGC GG ATC CAC ATG TCT TCG ACA

AGA CCC CAA) and CKGFPHIND with ligating into *BamH*I and *Hind*III sites of pSG1729. Fusions GFP-H160-R176 and GFP-R176-Y192 were created by deleting the corresponding parts from the construct pSG1729-GFP-C, using a QuikChange Mutagenesis kit (Stratagene). All constructed plasmid were then introduced into *B. subtilis* strain IS75 (*his leu met*) producing strains BD4316 (GFP-ComK), BD4317 (GFP-*K17*), BD4318 (GFP-R176-Y192) and BD4319 (GFP). In these strains *mecA* was disrupted by transformation with chromosomal DNA from BD2091 creating strains BD4320, BD4321, BD4322 and BD4323 respectively.

Cross-linking and AspN cleavage reactions

Peptides K17BB and S17BB were mixed with equimolar amounts of K17Scramble and MecA, and cross-linked as described in Experimental Procedures. (The K17Scramble peptide was added to reduce non-specific binding). A negative control sample, MecA with K17Scramble, was handled the same way. The cross-linked samples were loaded on a Ni-NTA Superflow (Qiagen) column and washed with 50 mM sodium phosphate, pH 7.4, 400 mM NaCl. Samples were eluted with 400 mM imidazole and dialyzed against PBS. A total of 200 μg of cross-linked protein was cleaved with 1 μg of Asp-N protease (Sigma) at 37°C for 18 hours. The digested protein samples were mixed with streptavidin Magnesphere paramagnetic beads (Promega) previously equilibrated with PBS, and the binding reaction was continued for 1 hour at room temperature. Each sample was washed extensively with 5 ml of 50 mM sodium phosphate pH 7.4, 400 mM NaCl and 30 ml of PBS. 150 μl of SDS loading buffer was then added and the mixtures were incubated at 70°C for 5 minutes. Elution samples were resolved using 16% tris-tricine polyacrylamide gels and electroblotted onto Immobilon Psq PVDF membrane (Millipore) as described. (Persuh et al., 1999). To detect cross-linking of the biotin labeled K17BB and S17BB to MecA, the cross-linked proteins were probed used anti-biotin mouse IgG monoclonal 2F5 (Molecular Probes), at a dilution of 1:2000. Cross-linked protein fragments were N-terminally sequenced by the Protein Chemistry Core Facility of Columbia University, NY, USA.

Table1. Strains

Strain	Genotype	Host strain	Source
BD4310	*PxylA-comK wt::amyE(cat), comk::spc, comG-lacZ(kan)*	*B.subtilis* IS75	This study
BD4311	*PxylA-comK (Δ M169-Y192)::amyE(cat), comk::spc, comG-lacZ (kan)*	*B.subtilis* IS75	This study
BD4312	*PxylA-comK (Δ E174-Y192)::amyE(cat), comG-lacZ(kan)*	*B.subtilis* IS75	This study
BD4313	*PxylA-comK wt::amyE(cat), comk::spc, ΔmecA(ery), comG-lacZ(kan)*	*B.subtilis* IS75	This study
BD4314	*PxylA-comK (Δ E174-Y192)::amyE, comk::spc, ΔmecA(ery), comG-lacZ(kan)*	*B.subtilis* IS75	This study
BD4315	*PxylA-comK (Δ M169-Y192)::amyE(cat), comk::spc, ΔmecA(ery), comG-lacZ (kan)*	*B.subtilis* IS75	This study
BD4316	*Pxyl-gfp-comK::amyE(spc)*	*B.subtilis* IS75	This study
BD4317	*Pxyl-gfp-comK(H160-R176)::amyE(spc)*	*B.subtilis* IS75	This study
BD4318	*Pxyl-gfp-comK(R176-Y192)::amyE(spc)*	*B.subtilis* IS75	This study
BD4319	*Pxyl-gfp::amyE(spc)*	*B.subtilis* IS75	This study
BD4320	*Pxyl-gfp-comK::amyE(spc), ΔmecA(ery)*	*B.subtilis* IS75	This study
BD4321	*Pxyl-gfp-comK(H160-R176)::amyE(spc), ΔmecA(ery)*	*B.subtilis* IS75	This study
BD4322	*Pxyl-gfp-comK(R176-Y192)::amyE(spc), ΔmecA(ery)*	*B.subtilis* IS75	This study
BD4323	*Pxyl-gfp::amyE(spc), ΔmecA(ery)*	*B.subtilis* IS75	This study
ED1034	pUB110::pT7T3 19U::*comS* (phleo)	*E.coli* DH5a	This study
BD4405	*Pxyl-gfp-comK(H160-R176)::amyE(spc), ΔcomS(cat), ΔcomK(kan)*	*B.subtilis* IS75	This study
BD4406	*Pxyl-gfp-comK(H160-R176)::amyE(spc), ΔcomK(kan), mc comS*-pUB110::pT7T3 19U::*comS* (phleo)	*B.subtilis* IS75	This study

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Fig. S1. GFP-K17 is degraded in vivo in the presence of MecA: microscopic confirmation. Strains expressing GFP-K17 in *mecA* and *mecA*$^{+}$ backgrounds were visualized by fluorescence and differential interference contrast (DIC) microscopy. Cells were prepared for microscopy and examined as described previously (Hahn et al., 2005).

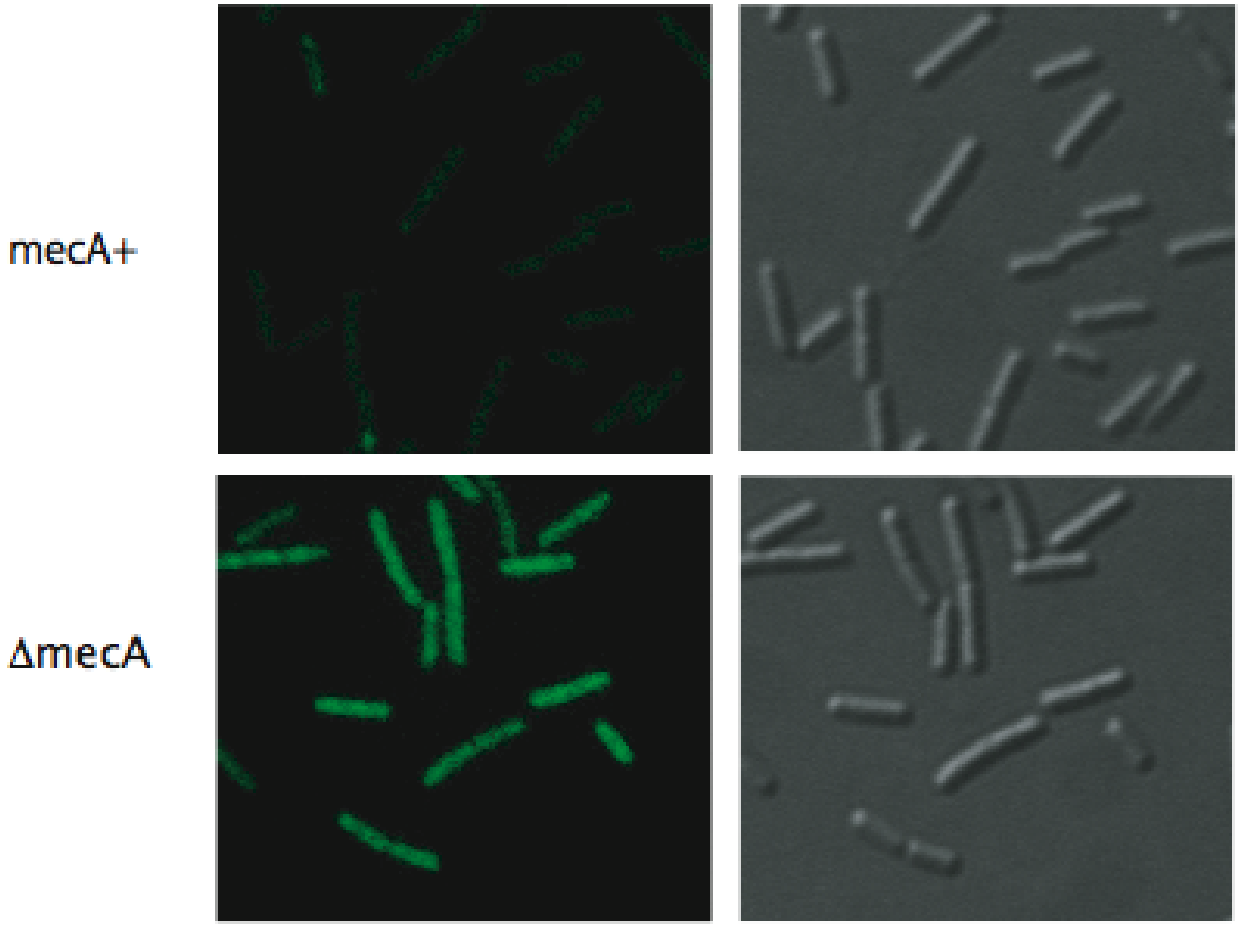

Fig. S2. Secondary structure prediction and alignment of ComK sequences. The secondary structure of the C-terminal 57 residues of ComK was predicted using Predict Protein (http://www.predictprotein.org) (Fig. S2A). The residues in the K17 peptide are in boldface and the minimal sequence for binding to MecA is underlined. Alignments of the C-terminal predicted sequences of ComK from the indicated species (using CLUSTAL W) are also shown (Fig. S2B). The residues involved in binding to MecA from *B. subtilis* ComK are in boldface as are the equivalent residues from *B. licheniformis*.

A

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136-SYNSFENQVYRTAWLRTKFQDRID**HRVPKRQEFMLYPKEER**TKMIYDFILRELGERY-192
    ----HHHHHHHHHHHHHHHHH---LLL---EEE-LLHHHH-HHHHHHHHHHH--LL
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B

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B. subtilis                 TFSNGKTMELPISYNSFENQVYRTAWLRTKFQDRID-HRVP**KRQEFMLYPK**EERTKMIYDFILRELGERY--- 192
B. licheniformis ATCC14580  TFVNGETLELPVSISSFENQVYRTAWLRTKFIDRIEGNPMQ**KKQEFMLYPK**EDRNQLIYEFILRELKKRY--- 192
G. kaustophilus HTA426      YFRNGKSISLDVSCKSFINQLYRTAQLRTKLSERMEARERKLQYIFRMQQEKQS------------------- 185
B. weihenstephanensis KBAB4 RFINGFAVKLEMSKSSFENQQNRTAKLRTEYEDRKKKQGNPCFKEIDKNEESKLTPAYEKVYFVKEGEV---- 193
B. cereus NVH 391-98        RFINGFAVKLEMSKSSFENQQNRTAKLRTEYEDRKDKQGNLQFKPVAKEAISTLRPAYEKVYLVKEEDIEGE- 196
O. iheyensis HTE831         LFKNGSKISLDISHGSLINQLQRTAQIRFLLDDRIKRWRKPPHSEDDKDS---------------------- 169
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