SUPPLEMENTAL MATERIAL

Dynamics of the peptidogylcan biosynthetic machinery in the stalked budding bacterium *Hyphomonas neptunium*

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SUPPLEMENTAL FIGURES

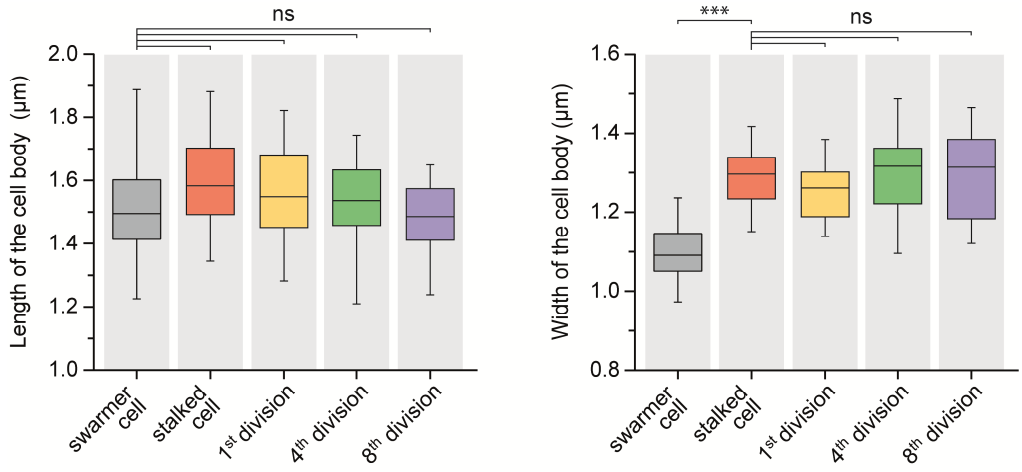

Figure S1. Morphological changes of *H. neptunium* over the course of multiple cell cycles. Swarmer cells were immobilized in a microfluidic flow cell and followed by time-lapse DIC microscopy at 15-min intervals. The lengths and widths of the (mother) cell bodies were measured immediately after immobilization (swarmer cell), right before the onset of bud formation (stalked cell), at the end of the first cell cycle immediately before the release of the mature bud (1st division), and at the end of the fourth and eighth cell cycle. The data are shown as box plots, with the bar indicating the median, the box the interquartile range, and the whiskers the 5th and 95th percentile. Significant differences are indicated by asterisks (***; t-test, $p < 10^{-8}$). The lack of significance (t-test; $p > 0.05$) is indicated by "ns". n = 31.

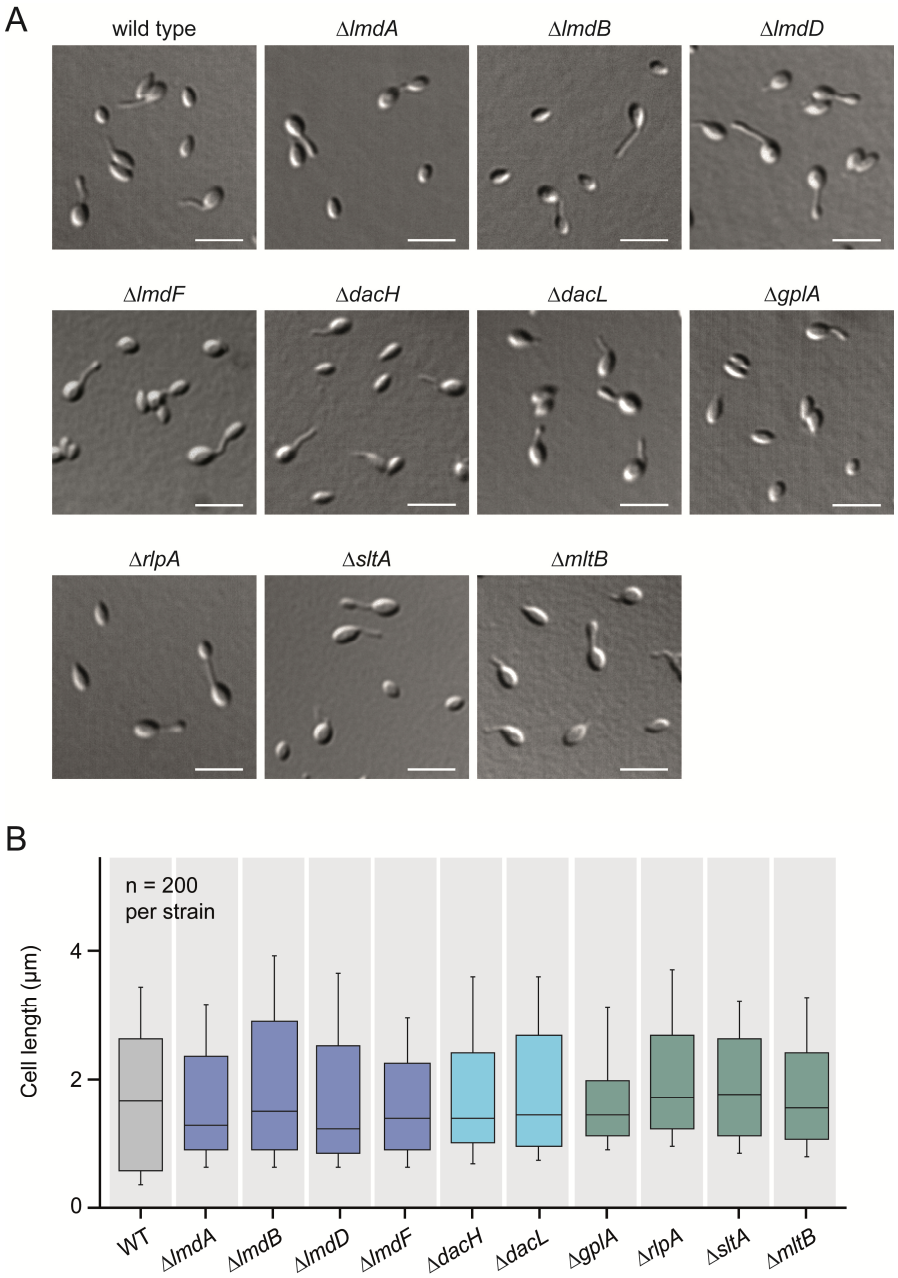

Figure S2. Morphology of PG hydrolase mutants. The *H. neptunium* wild type and strains EC36 (Δ*lmdA*), EC53 (Δ*lmdB*), EC38 (Δ*lmdD*), EC39 (Δ*lmdE*), EC90 (Δ*lmdF*), SR11 (Δ*dacB*), SR08 (Δ*dacH*), EC46 (Δ*dacL*), EC95 (Δ*mltA*), SR07 (Δ*gplA*), SR33 (Δ*rlpA*), EC21 (Δ*sltA*), SR20 (Δ*mltB*), and SR18 (Δ*amiC*) were grown to exponential phase and analyzed by DIC microscopy. Scale bar: 3 µm. **(B)** Cell lengths of the hydrolase mutant strains. The lengths of cells grown as described in (A) were quantified and shown as box plots, with the bar representing the median, the boxes the interquartile range, and the whiskers the 5th and 95th percentile. None of the distributions obtained for the mutant strains is significantly different from that of the wild-type strain (t-test, $p > 0.01$).

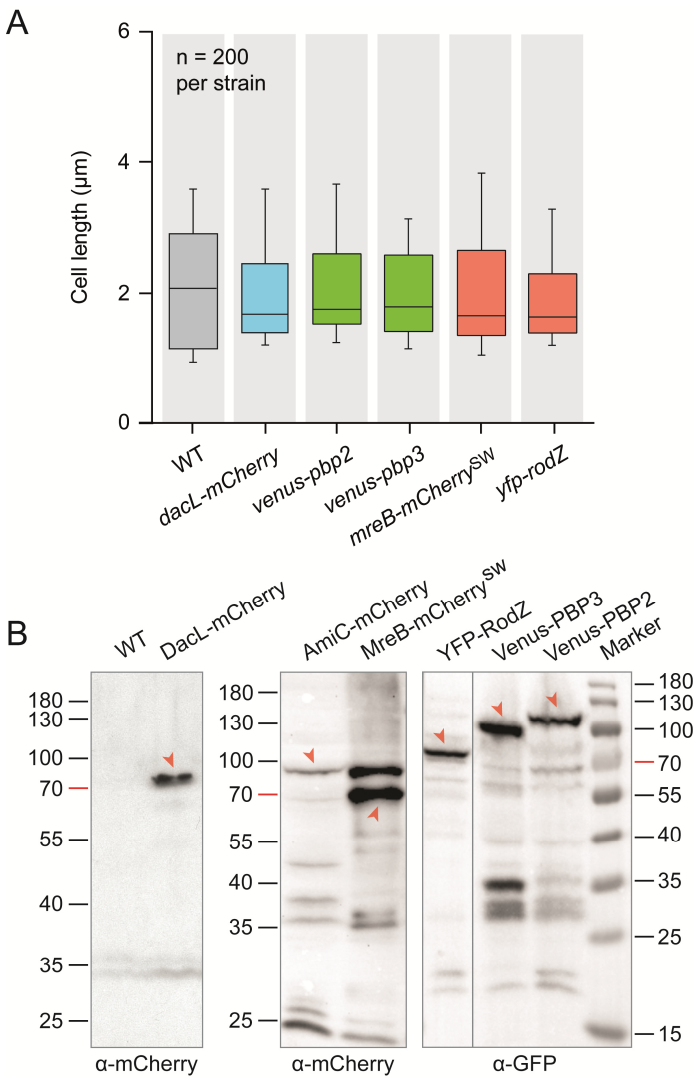

Figure S3. Characterization of strains producing fluorescently tagged PG biosynthetic proteins. (A) Cell length analysis. The *H. neptunium* wild type and strains SR28 (*dacL-mCherry*), SR14 (*venus-pbp2*), SE161 (*venus- pbp3*), EC63 (*mreB-mCherry*^{SW}), and EC93 (*yfp-rodZ*) were grown to exponential phase and imaged by DIC microscopy. The lengths of the cells were measured and shown in box plots, with the bar representing the median, the boxes the interquartile range, and the whiskers the 5th and 95th percentile. None of the distributions is significantly different from that of the wild-type strain (t-test, $p > 0.01$). **(B)** Stability of the fusion proteins analyzed in this study. The *H. neptunium* wild type and strains SR28 (*dacL-mCherry*), EC70 (P_{cu}::P_{cu}-*amiC-mCherry*), EC63 (*mreB-mCherry*^{SW}), EC93 (*yfp-rodZ*), SE161 (*venus-pbp3*), and SR14 (*venus-pbp2*) were grown to exponential phase and subjected to Western blot analysis with an anti-mCherry or anti-GFP antibody (performed as described by Jung *et al.*, 2015). Strain EC70 was induced for 24 h with 300 µM $CuSO_4$ before analysis. Arrows indicate the full-length fusion proteins.

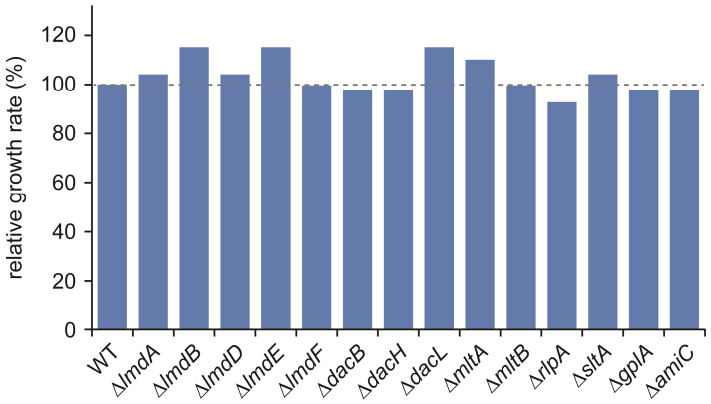

Figure S4. Growth rates of peptidoglycan hydrolase mutants. The growth of the *H. neptunium* wild type and the indicated PG hydrolase-deficient strains EC36 (Δ*lmdA*), EC53 (Δ*lmdB*), EC38 (Δ*lmdD*), EC39 (Δ*lmdE*), EC90 (Δ*lmdF*), SR11 (Δ*dacB*), SR08 (Δ*dacH*), EC46 (Δ*dacL*), EC95 (Δ*mltA*), SR20 (Δ*mltB*), SR33 (Δ*rlpA*), EC21 (Δ*sltA*), SR07 (Δ*gplA*), and SR18 (Δ*amiC*) was followed using a microplate reader. The doubling times were normalized to the value obtained for the wild-type strain (3.6 ± 0.3 h).

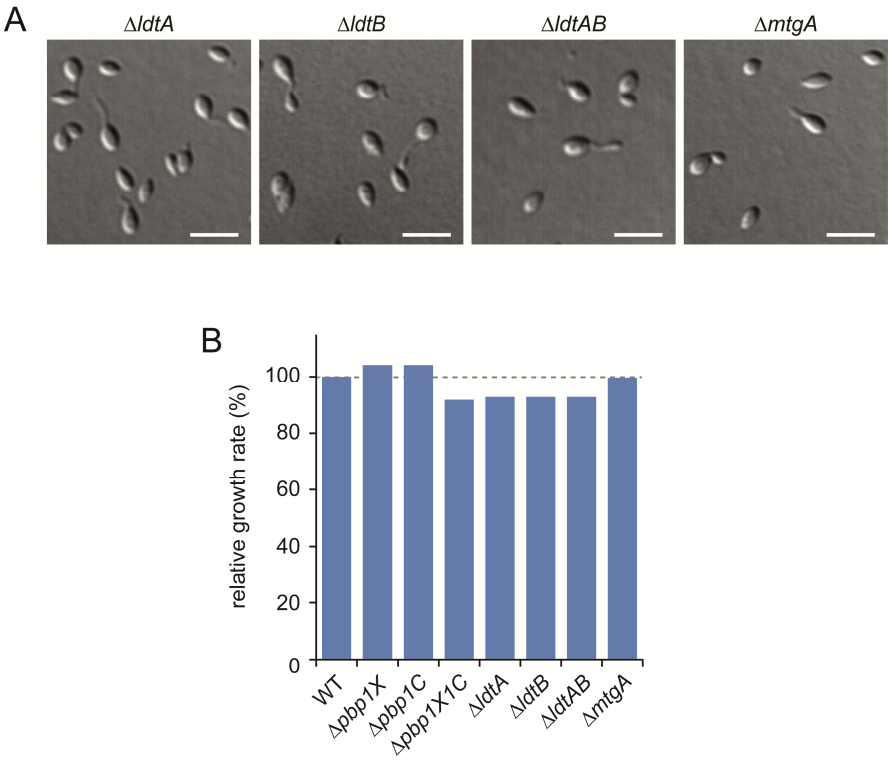

Figure S5. (A) Morphology of PG synthase mutants. Cells of strains SR31 (Δ*ldtA*), CR04 (Δ*ldtB*), SR32 (Δ*ldtAB*), and EC89 (Δ*mtgA*) were grown to exponential phase and analyzed by DIC microscopy. Scale bar: 3 µm. **(B)** Growth rates of PG synthase mutants. The growth of the *H. neptunium* wild type and the indicated PG synthase-deficient strains EC26 (*Δpbp1X*), EC27 (*Δpbp1C*), and EC57 (*Δpbp1X Δpbp1C*), SR31 (Δ*ldtA*), CR04 (Δ*ldtB*), SR32 (Δ*ldtAB*), and EC89 (Δ*mtgA*) was followed using a microplate reader. The doubling times were normalized to the value obtained for the wild-type strain (3.6 ± 0.3 h).

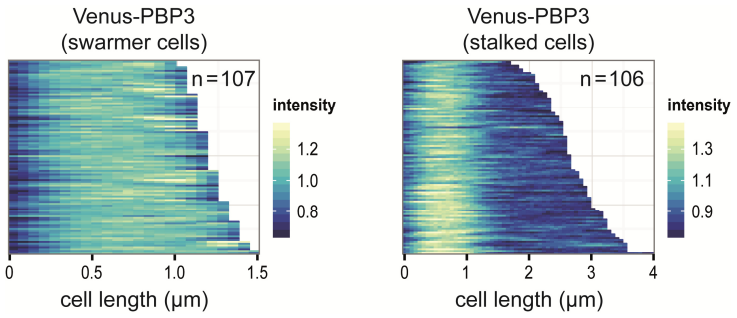

Figure S6. Subcellular distribution of Venus-PBP3. Shown are demographs of swarmer and stalked cells of strain SE161 (*venus-pbp3*).

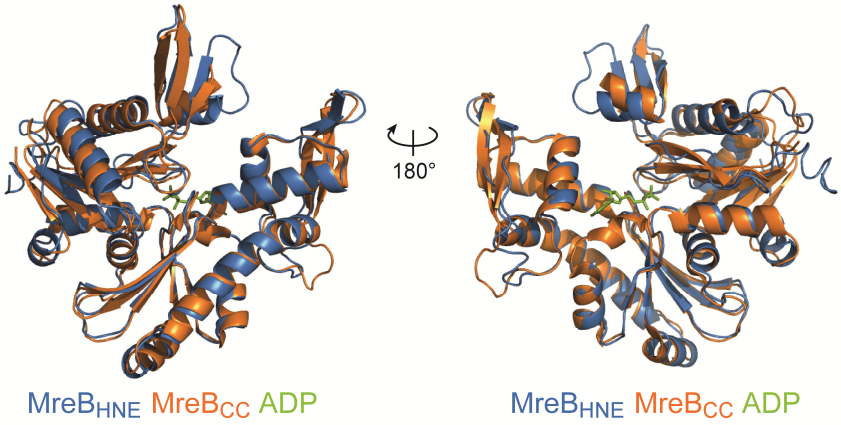

Figure S7. Structural model of *H. neptunium* MreB. Structural model of *H. neptunium* MreB (MreB$_{HNE}$; in blue), generated using the crystal structure of ADP-bound MreB from *C. crescentus* (MreB$_{CC}$; PDB 4CZF; in orange) as a template.

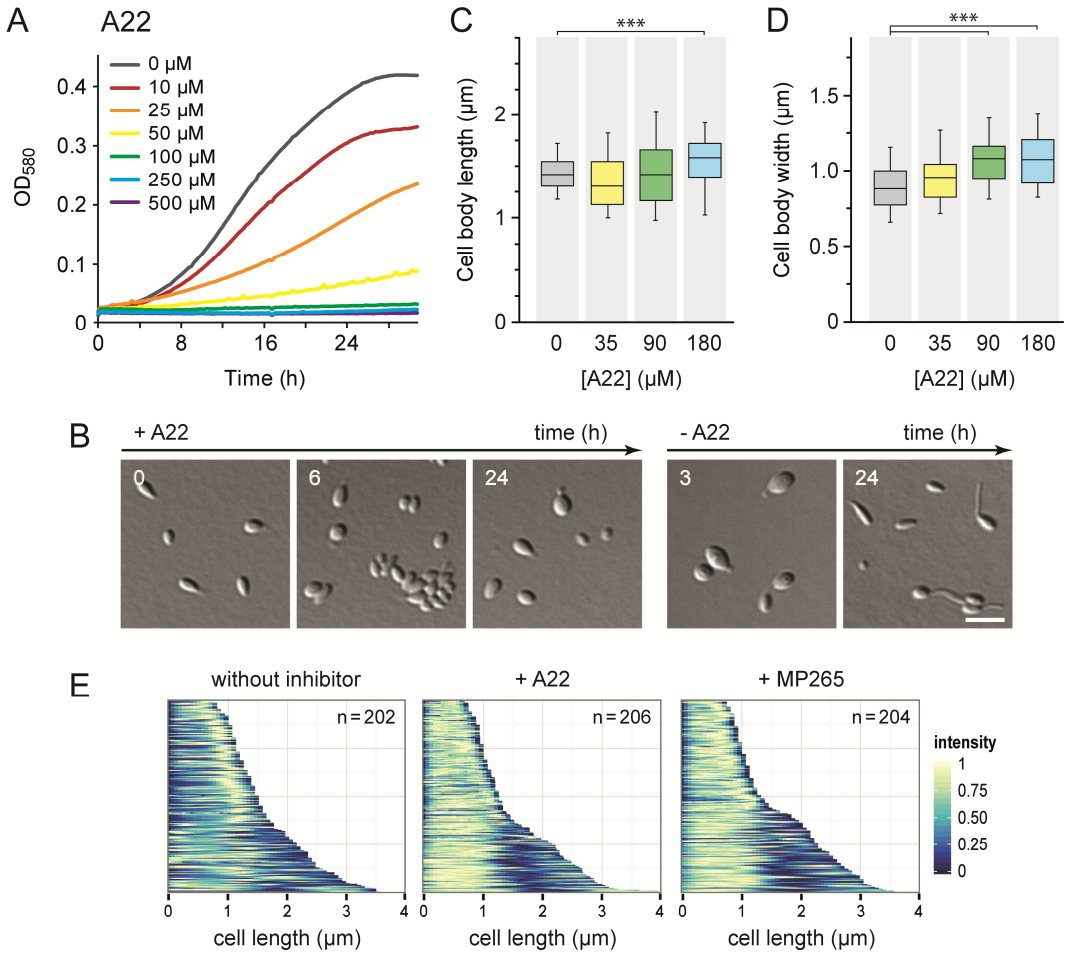

Figure S8. Effect of the MreB inhibitor A22 on MreB. (A) Inhibition of *H. neptunium* growth by the MreB inhibitor A22. Exponentially growing *H. neptunium* wild-type cells were diluted into fresh media containing the indicated concentrations of A22. Subsequently, their growth was monitored for 30 h using a microplate reader. **(B)** Effect of A22 on cell morphology. Cells were grown to exponential phase and exposed for 24 h to 90 µM A22. After washing and resuspension in medium lacking the inhibitor, cells were cultivated further to monitor the gradual recovery of wild-type morphology. Samples were taken at the indicated time points after addition (+) or removal (-) of A22 and analyzed by DIC microscopy. Scale bar: 3 µm. **(C,D)** Quantitative analysis of changes in cell shape after A22 treatment. Cells were grown to exponential phase and exposed to the indicated concentrations of A22 for 24 h. After imaging by DIC microscopy, the lengths (C) and widths (D) of their cell bodies (excluding stalks and buds) were measured. The data are shown as box plots, as described in Figure S3A. Distributions significantly different from that of the control culture (0 µM) are indicated (***; t-test, $p < 10^{-3}$ in C and $p < 10^{-12}$ in D). n = 100 for each concentration. **(E)** Delocalization of MreB-mCherry^{SW} in the presence of A22 or MP265. Cells of strain EC63 (*mreB-mCherry*^{SW}) were grown to exponential phase, exposed to A22 (35 µM) or MP265 (250 µM) for 1 h, and subjected to fluorescence microscopy. After determination of the fluorescence profiles of random subpopulations of cells, the data were plotted as demographs. Cells grown in the absence of any inhibitor are analyzed as a control.

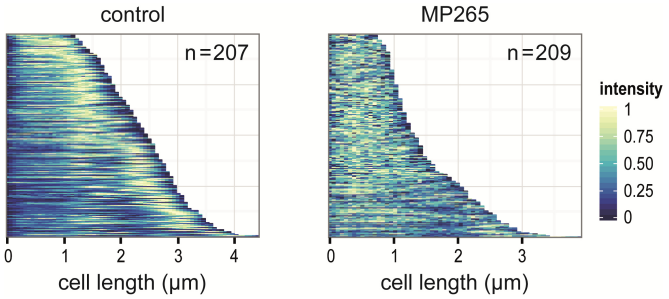

Figure S9. Delocalization of PG incorporation after inhibition of MreB. Cells of the *H. neptunium* wild-type strain were grown for 5 h in the absence (control) or presence of 250 µM MP265, labeled with HADA, and analyzed by fluorescence microscopy. The demographs show fluorescence profiles obtained from random subpopulations of cells from each condition.

SUPPLEMENTAL MOVIES

Movie S1. Time-lapse analysis of growing *H. neptunium* cells. Wild-type cells were grown in a microfluidic device and imaged at 15 min intervals by DIC microscopy. Scale bar: 2 µm.

SUPPLEMENTAL TABLES

Table S1. Proteins investigated in this work.

Protein	ORF number	Predicted function
Lytic enzymes		
Metallopeptidases		
LmdA	HNE_0632	Peptidase (M23 family)
LmdB	HNE_0633	Peptidase (M23 family)
LmdC	HNE_2628	Peptidase (M23 family)
LmdD	HNE_2982	Peptidase (M23 family)
LmdE	HNE_3210	Peptidase (M23 family)
LmdF	HNE_3409	Peptidase (M23 family)
Carboxypeptidases		
DacB	HNE_0402	D-Ala-D-Ala carboxy-/endopeptidase
DacH	HNE_1025	D-Ala-D-Ala carboxypeptidase
DacL	HNE_1814	D-Ala-D-Ala carboxypeptidase
Glycosyl hydrolases		
GplA	HNE_0445	Glycosyl hydrolase family protein
MltA	HNE_0008	Lytic murein transglycosylase MltA
MltB	HNE_3349	Lytic murein transglycosylase
RlpA	HNE_1815	Lytic transglycosylase
SltA	HNE_2801	Lytic transglycosylase, SLT family
Amidase		
AmiC	HNE_0674	N-acetylmuramoyl-L-Ala amidase
Synthetic enzymes		
Bifunctional PBPs		
PBP1A	HNE_1911	Penicillin-binding protein 1A
PBP1C	HNE_3002	Penicillin-binding protein 1C
PBP1X	HNE_0768	Penicillin-binding protein, 1A family
Monofunctional PBPs		
PBP2	HNE_2934	Penicillin-binding protein 2
PBP3	HNE_3030	Penicillin-binding protein 3, FtsI
L,D-Transpeptidases		
LdtA	HNE_0929	L,D-transpeptidase
LdtB	HNE_3551	L,D-transpeptidase
Synthetic Transglycosylase		
MtgA	HNE_3102	Monosynthetic transglycosylase
Regulatory factors		
MreB	HNE_2937	Actin homologue MreB
RodZ	HNE_0620	MreB-associated protein RodZ

Table S2. ***H. neptunium*** **strains used in this study.**

Strains	Genotype/description	Construction	References
LE670	Wild type (ATCC 15444)		Leifson (1964)
CR04	LE670 *ΔHNE3551 (ldtB)*	In-frame deletion of HNE3551 in LE670 using pCR04	This study
EC21	LE670 *ΔHNE2801 (sltA)*	In-frame deletion of HNE2801 in LE670 using pEC28	This study
EC26	LE670 *ΔHNE0768 (pbp1X)*	In-frame deletion of HNE0768 in LE670 using pEC22	This study
EC27	LE670 *ΔHNE3002 (pbp1C)*	In-frame deletion of HNE3002 in LE670 using pEC26	This study
EC36	LE670 *ΔHNE0632 (lmdA)*	In-frame deletion of HNE0632 in LE670 using pEC34	This study
EC38	LE670 *ΔHNE2982 (lmdD)*	In-frame deletion of HNE2982 in LE670 using pEC38	This study
EC39	LE670 *ΔHNE3210 (lmdE)*	In-frame deletion of HNE3210 in LE670 using pEC39	This study
EC46	LE670 *ΔHNE1814 (dacL)*	In-frame deletion of HNE1814 in LE670 using pEC64	This study
EC53	LE670 *ΔHNE0633 (lmdB)*	In-frame deletion of HNE0633 in LE670 using pEC35	This study
EC57	LE670 *ΔHNE0768 (pbp1X) ΔHNE3002 (pbp1C)*	In-frame deletion of HNE3002 in EC26 using pEC26	This study
EC63	*LE670 mreB-mCherry*^{SW}	Gene replacement in LE670 using pEC87	This study
EC70	LE670 P_{cu}::P_{cu}-*HNE0674 (amiC)-mCherry*	Integration of pEC115 in LE670	This study
EC89	LE670 *ΔHNE3102 (mtgA)*	In-frame deletion of HNE3102 in LE670 using pEC65	This study
EC90	LE670 *ΔHNE3409 (lmdF)*	In-frame deletion of HNE3409 in LE670 using pEC126	This study
EC93	LE670 *yfp-HNE0620 (rodZ)*	Gene replacement in LE670 using pEC129	This study
EC95	LE670 *ΔHNE0008 (mltA)*	In-frame deletion of HNE0008 in LE670 using pEC157	This study
SE161	LE670 *venus-HNE3030* (*pbp3*)	Gene replacement in LE670 using pSE68	This study
SR07	*LE670 ΔHNE0445 (glpA)*	In-frame deletion of HNE0445 in LE670 using pSR02	This study
SR08	LE670 *ΔHNE1025 (dacH)*	In-frame deletion of HNE1025 in LE670 using pSR03	This study
SR11	LE670 *ΔHNE0402 (dacB)*	In-frame deletion of HNE0402 in LE670 using pSR01	This study
SR14	LE670 *venus-HNE2934 (pbp2)*	Gene replacement in LE670 using pSR06	This study
SR18	LE670 *ΔHNE0674 (amiC)*	In-frame deletion of HNE0674 in LE670 using pSR22	This study
SR20	LE670 *ΔHNE3349 (mltB)*	In-frame deletion of HNE3349 in LE670 using pSR19	This study
SR24	LE670 *HNE0633 (lmdB)-mCherry*	Gene replacement in LE670 using pSR13	This study
SR26	LE670 *HNE3409 (lmdF)-mCherry*	Gene replacement in LE670 using pSR17	This study
SR28	LE670 *HNE1814 (dacL)-mCherry*	Gene replacement in LE670 using pSR38	This study
SR31	LE670 *ΔHNE0929 (ldtA)*	In-frame deletion of HNE0929 in LE670 using pEC174	This study
SR32	LE670 *ΔHNE0929 (ldtA) ΔHNE3551 (ldtB)*	In-frame deletion of HNE0929 in CR04 using pEC174	This study
SR33	LE670 *ΔHNE1815 (rlpA)*	In-frame deletion of HNE1815 in LE670 using pEC172	This study

Table S3. *E. coli* strains used in this study.

Strains	Genotype/description	References
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
WM6034	*thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4–1360 Δ(*araBAD)567 ΔdapA*1341::[*erm pir*(wt)]	W. Metcalf (unpublished)

Table S4. General plasmids used in this work.

Plasmids	Description	References
pCCHYC-2	Integrating plasmid to construct C-terminal fusions to mCherry under the control of P_{cu}, Kan^R	Jung *et al.*, 2014
pCHYC-2	Integrating plasmid to construct C-terminal fusions to mCherry at a site of interest, Kan^R	Thanbichler *et al.*, 2007
pCVENC-2	Integrating plasmid to construct C-terminal fusions to Venus under the control of P_{cu}, Kan^R	Jung *et al.*, 2014
pCVENN-3	Integrating plasmid to construct N-terminal fusions to Venus under the control of P_{cu}, Rif^R	Jung *et al.*, 2014
pCYFPC-2	Integrating plasmid to construct C-terminal fusions to YFP under the control of P_{cu}, Kan^R	Jung *et al.*, 2014
pCYFPN-2	Integrating plasmid to construct N-terminal fusions to YFP under the control of P_{cu}, Kan^R	Jung *et al.*, 2014
pNPTS138	*sacB*-containing suizide vector used for double homologous recombination, Kan^R	M.R. Alley, unpublished
pZVENN-2	Integrating plasmid to construct N-terminal fusions to Venus under the control of P_{zn}, Kan^R	Jung *et al.*, 2014

Table S5. Plasmids generated in this work.

Plasmids	Description	Construction
pCR04	pNPTS135 derivative for in-frame deletion of *HNE3551* (*ldtB*)	a) amplification of the HNE3551 flanking regions from LE670 chrom. DNA using primers oCR16+oCR17 (upstream) and oCR18+oCR19 (downstream), b) restriction of the upstream fragment with HindIII and BamHI, restriction of the downstream fragment with BamHI and NheI c) triple ligation with pNPTS138 cut with PstI and NheI
pEC115	pCCHYC-2 bearing *HNE0674 (amiC)-mCherry*	a) amplification of HNE_0674 from LE670 chrom. DNA using primers oEC157+SS271, restriction with NdeI and KnpI, b) ligation with pCCHYC-2 cut with NdeI and KnpI
pEC12	pCCHYC-2 bearing HNE3409 *(lmdF)-mCherry*	a) amplification of HNE3409 from LE670 chrom. DNA using primers oEC23+oEC24, restriction with KpnI and NdeI b) ligation with pCCHYC-2 cut with KpnI and NdeI
pEC126	pNPTS135 derivative for in-frame deletion of *HNE3409* (*lmdF)*	a) amplification of the HNE3409 flanking regions from LE670 chrom. DNA using primers oEC289+oEC290 (upstream) and oEC291+oEC292 (downstream), b) restriction of the upstream fragment with HindIII and BamHI, restriction of the downstream fragment with BamHI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pEC129	pNPTS135 derivative for replacing *rodZ* with *yfp-rodZ (HNE0620)*	a) amplification of three fragments for extension overlap PCR using primers oEC244+oEC245 (template: LE670 chrom. DNA), oEC246+oEC247 (template: pCYFPC-2), oEC248+oEC249 (template: LE670 chrom. DNA) b) fusion of the three fragments by extension-overlap PCR to generate upstream-*eyfp*-HNE0620 using primers oEC244+oEC249 c) restriction of upstream-*eyfp*-HNE0620 with HindIII and NheI and ligation with pNPTS138 cut with HindIII and NheI
pEC157	pNPTS135 derivative for in-frame deletion of *HNE0008 (mltA)*	a) amplification of the HNE0008 flanking regions from LE670 chrom. DNA using primers oEC313+oEC314 (upstream) and oEC315+oEC316 (downstream), b) restriction of the upstream fragment with HindIII and BamHI, restriction of the downstream fragment with BamHI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pEC172	pNPTS135 derivative for in-frame deletion of *HNE1815 (rlpA)*	a) amplification of the HNE1815 flanking regions from LE670 chrom. DNA using primers oEC326+oEC327 (upstream) b) amplification of the downstream region from LE670 chrom. DNA by a nested PCR using first primers oEC326+oE331 and then primers oEC328+oEC329 c) restriction of the upstream flank with BamHI and HindIII, restriction of downstream flank with BamHI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pEC174	pNPTS135 derivative for in-frame deletion of *HNE0929* (*ldtA*)	a) amplification of HNE0929 flanking regions from LE670 chrom. DNA using primers oEC338+oEC339 (upstream) and oEC340+oEC341 (downstream) b) restriction of upstream flank with BamHI and KpnI, restriction of downstream flank with KpnI and NheI c) triple ligation with pNPTS138 digested with BamHI and NheI
pEC22	pNPTS135 derivative for in-frame deletion of *HNE0768* (*pbp1X*)	a) amplification of HNE0768 flanking regions from LE670 chrom. DNA using primers oEC33+oEC34 (upstream) and oEC35+oEC36 (downstream), b) restriction of upstream fragment with PstI and HindIII, restriction of the downstream fragment with HindIII and NheI c) triple ligation with pNPTS138 cut with PstI and NheI
pEC26	pNPTS135 derivative for in-frame deletion of *HNE3002* (*pbp1C*)	a) amplification of the HNE3002 flanking regions from LE670 chrom. DNA using primers oEC49+oEC50 (upstream) and oEC51+oEC52 (downstream), b) restriction of the upstream fragment with EcoRI and HindIII, restriction of the downstream fragment with HindIII and NheI c) triple ligation with pNPTS138 cut with EcoRI and NheI
pEC28	pNPTS135 derivative for in-frame deletion of *HNE2801 (sltA)*	a) amplification of the HNE2801 flanking regions from LE670 chrom. DNA using primers oEC55+oEC56 (upstream) and oEC57+oEC58 (downstream) b) restriction of the upstream fragment with HindIII and NheI, restriction of downstream fragment with NheI and EcoRI c) triple ligation with pNPTS138 cut with HindIII and EcoRI
pEC34	pNPTS135 derivative for in-frame deletion of *HNE0632* (*lmdA)*	a) amplification of the HNE0632 flanking regions from LE670 chrom. DNA using primers oEC84+oEC85 (upstream) and oEC86+oEC87 (downstream) b) restriction of the upstream fragment with BamHI and HindIII, restriction of the downstream fragment with BamHI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI

Table S5. Plasmids generated in this work (continued).

pEC35	pNPTS135 derivative for in-frame deletion of *HNE0633* (*lmdB*)	a) amplification of the HNE0663 flanking regions from LE670 chrom. DNA using primers oEC88+oEC89 (upstream) and oEC90+oEC91 (downstream), b) restriction of the upstream fragment with PstI and HindIII, restriction of the downstream fragment with HindIII and NheI c) triple ligation with pNPTS138 cut with PstI and NheI
pEC38	pNPTS135 derivative for in-frame deletion of *HNE2982* (*lmdD)*	a) amplification of the HNE2982 flanking regions from LE670 chrom. DNA using primers oEC92+oEC93 (upstream) and oEC94+oEC95 (downstream) b) restriction of the upstream fragment with PstI and HindIII, restriction of the downstream fragment with HindIII and NheI c) triple ligation with pNPTS138 cut with PstI and NheI
pEC39	pNPTS135 derivative for in-frame deletion of *HNE3210* (*lmdE*)	a) amplification of the HNE3210 flanking regions from LE670 chrom. DNA using primers oEC96+oEC97 (upstream) and oEC98+oEC99 (downstream), b) restriction of the upstream fragment with EcoRI and HindIII, restriction of the downstream fragment with HindIII and NheI c) triple ligation with pNPTS138 cut with EcoRI and NheI
pEC48	pCCHYN-2 bearing *mCherry-mreB*	a) amplification of HNE_2937 from LE670 chrom. DNA using primers oEC123+oEC124, restriction with KpnI and NheI b) ligation with pCCHYN-2 cut with KpnI and NheI
pEC55	pCVENN-3 bearing *venus-HNE2934 (pbp2)*	a) amplification of HNE2934 from LE670 chrom. DNA using primers oEC9+oEC10, restriction with KpnI and NheI b) ligation into pCCHYN-2 cut with KpnI and NheI
pEC64	pNPTS135 derivative for in-frame deletion of *HNE1814 (dacL)*	a) amplification of HNE1814 flanking regions from LE670 chrom. DNA using primers oEC137+oEC138 (upstream) and oEC139+oEC140 (downstream) b) restriction of upstream flankwith EcoRI and PstI, restriction of downstream flank with EcoRI and NheI c) triple ligation with pNPTS138 cut PstI and NheI
pEC65	pNPTS135 derivative for in-frame deletion of *HNE3102 (mtgA)*	a) amplification of the HNE3102 flanking regions from LE670 chrom. DNA using primers oEC295+oEC142 (upstream) and oEC143+oEC296 (downstream), b) restriction of the upstream fragment with HindIII and EcoRI, restriction of the downstream fragment with EcoRI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pEC7	pCCHYC-2 bearing HNE1814 *(dacL)-mCherry*	a) amplification of HNE1814 from LE670 chrom. DNA using primers oEC27 and oEC28, restriction with KpnI and NdeI b) ligation into pCCHYC-2 cut with KpnI and NdeI
pEC87	pNPTS135 derivative for replacing *mreB* with *mreB-mCherry*^{SW}	a) amplification of three fragments for extension overlap PCR using primers oEC182+oEC184 (template pEC48), oEC183+oEC186 (template pCCHYC-2), oEC185+oEC124 (template pEC48) b) fusion of the fragments by extension-overlap PCR to generate an *mreB'-mCherry-'mreB* fragment using primers oEC182+oEC124 c) digestion of *mreB'-mCherry-'mreB* with HindIII and NheI and ligation into pNPTS138 cut with HindIII and NheI
pSE68	pNPTS138 derivative for replacing native *ftsI* with *venus-ftsI (HNE3030)*	a) amplification of the upstream region of *HNE3030* from ATCC15444 chrom. DNA using primers oSE88+oSE89 b) amplification of HNE3030 from LE670 chrom. DNA using primers oSE20+oSE21, restriction with KpnI and SacI, ligation into pZVENN-2 cut with KpnI and SacI, amplification of *venus*-ftsI' from the resulting plasmid using primers oSE90+oSE91 c) fusion of the two PCR-products by overlap-extension PCR d) digestion with HindIII and BamHI and ligation into pNPTS138 cut with HindIII BamHI
pSR01	pNPTS135 derivative for in-frame deletion of *HNE0402 (dacB)*	a) amplification of the HNE0402 flanking regions from LE670 chrom. DNA using primers oSR01+oSR02 (upstream) and oSR03+oSR04 (downstream) b) restriction of the upstream fragment with HindIII and EcoRI, restriction of the downstream fragment with EcoRI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pSR02	pNPTS135 derivative for in-frame deletion of *HNE0445 (gplA)*	a) amplification of the HNE0445 flanking regions from LE670 chrom. DNA using primers oSR05+oSR06 (upstream) and oSR07+oSR08 (downstream) b) restriction of the upstream fragment with HindIII and EcoRI, restriction of the downstream fragment with EcoRI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI
pSR03	pNPTS135 derivative for in-frame deletion of *HNE1025 (dacH)*	a) amplification of the HNE1025 flanking regions from LE670 chrom. DNA using primers oSR9+oSR10 (upstream) and oSR11+oSR12 (downstream) b) restriction of the upstream fragment with HindIII and EcoRI, restriction of the downstream fragment with EcoRI and NheI c) triple ligation with pNPTS138 cut with HindIII and NheI

Table S5. Plasmids generated in this work (continued).

pSR06	pNPTS138 derivative for replacing native *HNE2934* with *venus-HNE2934 (pbp2)*	a) amplification of two fragments for extension overlap PCR using primers oSR32+oSR31 (template: pEC55) and oEC48+oEC33 (template: LE670 chrom. DNA) b) fusion of both fragments by extension-overlap PCR to generate upstream-*venus-pbp2* using oEC48+oEC31 c) restriction of upstream-*venus*-HNE2934 with HindIII and NheI and ligation with pNPTS138 digested with HindIII and NheI
pSR17	pNPTS138 derivative for replacing native *HNE3409* with *HNE3409 (lmdF)-mCherry*	a) amplification of two fragments for extension overlap PCR using primers oSR70+oSR72 (template: pEC12) and oSR71+oEC103 (template: LE670 chrom. DNA) b) fusion of both fragments by extension overlap PCR to generate HNE3409-*mCherry*-downstream using primers oSR70+oEC103 c) restriction of HNE3409-*mCherry*-downstream with HindIII and NheI and ligation with pNPTS138 digested with HindIII and NheI
pSR19	pNPTS135 derivative for in-frame deletion of *HNE3349 (mltB)*	a) amplification of two fragments for extension overlap PCR using primers oSR81+oSR84 (template: pCVENC-2) and oSR82+oSR83 (template LE670 chrom. DNA) b) fusion of both fragments by extension overlap PCR to generate HNE3349-*mCherry*-downstream using primers oSR81+oSR82 c) digestion of HNE3349-*mCherry*-downstream with HindIII and NheI and ligation with pNPTS138 cut with HindIII and NheI
pSR22	pNPTS135 derivative for in-frame deletion of *HNE0674 (amiC)*	a) amplification of the HNE0674 flanking regions from LE670 chrom. DNA using primers oSR89+oSR90 (upstream) and oSR91+oSR92 (downstream) b) restriction of the upstream fragment with HindIII and BamHI, restriction of the downstream fragment with BamHI and NheI c) triple ligation with pNPTS138 digested with HindIII and NheI
pSR38	pNPTS138 derivative for replacing native *HNE1814* with *HNE1814 (dacL)-mCherry*	a) amplification of two fragments for extension overlap PCR using primers oSR73+oSR76 (template: pEC07) and oSR74+oSR75 (template: LE670 chrom. DNA) b) fusion of both fragments by extension overlap PCR to generate HNE1814-*mCherry*-downstream using primers oS73+oSR74 c) restriction of HNE1814-*mCherry*-downstream with HindIII and NheI and ligation with pNPTS138 cut with HindIII and NheI

Table S6. Oligonucleotides used in this work.

ID	Oligonucleotide	Sequence [1]	Restriction site
oCR16	HNE_3551_Del1f	TTTT**AAGCTT**GTATGGGTTGTGACGGCCGCG	HindIII
oCR17	HNE_3551_Del1r	CTA**GGATCC**CAGTTTTCCCTCTGAAAAGGC	BamHI
oCR18	HNE_3551_Del2f	GAG**GGATCC**CGTCTGACGGGCCGTTCGGCG	BamHI
oCR19	HNE_3551_Del2r	TATA**GCTAGC**GCTTTCTCGCTCATCAAGATG	NheI
oEC9	HNE_2934_for	AAAA**GGTACC**ATGAGCCGGAAAAGTAAAAACGCTG	KpnI
oEC10	HNE_2934_rev	TTTT**GCTAGC**TCATGCCGGGTCACCGGTGTTG	NheI
oEC23	HNE_3409_for	AAA**CATATG**CTGAAAAGACGCTTATCCGCC	NdeI
oEC24	HNE_3409_rev	TTT**GGTACC**TTCGATGATCTCGTAGCCTTCGGG	KpnI
oEC27	HNE_1814_for	TTT**CATATG**GTGTTTGCGGCCCTACTGACCCTCG	NdeI
oEC28	HNE_1814_rev	ATA**GGTACC**GTCATTGCTGCTGGAAATAAACGTG	KpnI
oEC33	HNE_0768_del1	TAT**CTGCAG**CACGAAGCCCGGCATGTCCTCAT	PstI
oEC34	HNE_0768_del2	TAT**AAGCTT**GGGTGAAGGCACCCGCTGGTATA	HindIII
oEC35	HNE_0768_del3	ATAT**AAGCTT**CAGGCGGAATAGGCCAAAGAACAG	HindIII
oEC36	HNE_0768_del4	ATAT**GCTAGC**GCTATGCGTGGCGATGGCGGACCT	NheI
oEC49	HNE_3002_del1	AAA**GAATTC**GGATCAATGCCGCGAATGAAGTGGG	EcoRI
oEC50	HNE_3002_del2	TTT**AAGCTT**GACCCCGCCTGACGCCCGGTCTG	HindIII
oEC51	HNE_3002_del3	TTTT**AAGCTT**GCGTTTCGGCACGGACTGGCCC	HindIII
oEC52	HNE_3002_del4	TTTT**GCTAGC**GCGCTTCCTCGGCATGGACGGC	NheI
oEC55	HNE_2801_del1	ATAT**AAGCTT**TATAAAGCACCACGGGCAGGGCGAC	HindIII
oEC56	HNE_2801_del2	ATAT**GCTAGC**AATCCGGAGCATGGTTCGGCT	NheI
oEC57	HNE_2801_del3	ATAT**GCTAGC**GGCCGCCGCTGACCGGAAGTGTC	NheI
oEC58	HNE_2801_del4	ATAT**GAATTC**GTAAGCACGCCAATGGTCGCCGA	EcoRI
oEC84	HNE_0632_del1	TAT**AAGCTT**GGTGTCCGAGCAGGCCCGCGAGCAT	HindIII
oEC85	HNE_0632_del2	ATA**GGATCC**AACTCCGCCCGGATGGCAAACGCCC	BamHI
oEC86	HNE_0632_del3	ATA**GGATCC**GACAGGAATCCGAGGCAGCTTTCGC	BamHI
oEC87	HNE_0632_del4	TTTT**GCTAGC**GACACCGCCTATGCCCACCTCTCGC	NheI
oEC88	0633_del1new	TAT**CTGCAG**GGCGCAGGCGCTGGCCCCGCGTGGG	PstI
oEC89	HNE_0633_del2	TAT**GAATTC**CCGCGTGAAGCAACGCCCGTAAGCC	EcoRI
oEC90	HNE_0633_del3	TAT**GAATTC**GGTTGCCGGGGCGATGGGAAGGTCG	EcoRI
oEC91	HNE_0633_del4	TTTT**GCTAGC**CTTCCTGGGCCTCTGCGGGCACATC	NheI
oEC92	HNE_2982_del1	TAT**CTGCAG**TCTATCAGGAAGACGGCAAGGTTTG	PstI
oEC93	HNE_2982_del2	TAT**AAGCTT**CCCTATGAAGGGGCGTTGCAGGCCG	HindIII
oEC94	HNE_2982_del3	TAT**AAGCTT**GGCCGCGAGCGCAAGAGCAATCGCC	HindIII
oEC95	HNE_2982_del4	TTTT**GCTAGC**CATTCTTCCCAAGCCCGGCATTGAC	NheI
oEC96	HNE_3210_del1	TAT**GAATTC**GGCCGTTGATCTCGGTGATATAGTC	EcoRI
oEC97	HNE_3210_del2	TAT**AAGCTT**CTTAATCCTGCAGAGTGGTTGTCGC	HindIII
oEC98	HNE_3210_del3	TAT**AAGCTT**AGGGCGGAAAAAGCGCATGCGCGGA	HindIII
oEC99	HNE_3210_del4	TTTT**GCTAGC**GGGCGTTGGCGTTGGGTGGCGCTTG	NheI
oEC103	HNE_3409_del4	TTTT**GCTAGC**TCGGTTGCGGGCGACAGCGCCGTTG	NheI
oEC123	2937_for	TTT**GGTACC**ATGATCGGTAGCCTCCTCGGCCTG	KpnI
oEC124	2937_rev	TTTT**GCTAGC**TCAGACTTCCGGGCAGAGGACGC	NheI
oEC137	HNE_1814_del1	TAT**CTGCAG**GCTTTTGGTCTGCGCTCGAACGTG	PstI
oEC138	HNE_1814_del2	ATAT**GAATTC**GTTCGCACGTTTATTTCCAGCAGC	EcoRI
oEC139	HNE_1814_del3	ATAT**GAATTC**GAGCGCTCCGAGGGTCAGTAGGGC	EcoRI
oEC140	HNE_1814_del4	TATA**GCTAGC**CGTGTCGGCGCCGGTTGCTGC	NheI
oEC142	HNE_3102_del2	ATAT**GAATTC**GTAGGCGGGGGCGGAGGAGTTCATG	EcoRI
oEC143	HNE_3102_del3	ATAT**GAATTC**CCACCGGCCCCACAGAACTCCGCC	EcoRI
oEC157	HNE_0674_for	TATA**CATATG**GGCACTCAACGCCCGTCTCATAC	NdeI
oEC182	2937_for2	TAT**AAGCTT**ATGATCGGTAGCCTCCTCGGCCTG	HindIII
oEC183	2937-SF-eol-for	CCGCGATGCCGCCTGAAAGCGGATCGAGCATGGTGAGCAAGGGCGAGGAG	-
oEC184	2937-SF-eol-rev	CTCCTCGCCCTTGCTCACCATGCTCGATCCGCTTTCAGGCGGCATCGCGG	-
oEC185	2937-SF-eol-for2	GGACGAGCTGTACAAGTCTGGTGCACCGGGTAACGGCACCGGCATGGCGC	-
oEC186	2937-SF-eol-rev2	GCGCCATGCCGGTGCCGTTACCCGGTGCACCAGACTTGTACAGCTCGTCC	-
oEC244	HNE_0620_eol_for	TATA**GCTAGC**CGCGCTCGACCATAAAGGT	NheI
oEC245	HNE_0620_eol_rev	TCCTCGCCCTTGCTCACCATATTCTACCAGTCACTTCGAC	-
oEC246	HNE_0620_eol_for2	GTCGAAGTGACTGGTAGAATATGGTGAGCAAGGGCGAGGA	-
oEC247	HNE_0620_eol_rev2	TGGGTCATGTTGTGTGCCATATGCATATTAATTAAGGCGC	-
oEC248	HNE_0620_eol_for3	GCGCCTTAATTAATATGCATATGGCACACAACATGACCCA	-
oEC249	HNE_0620_eol_rev3	TTT**AAGCTT**CGGCCAGTGTGCGGCTGAG	HindIII
oEC289	HNE_3409_del1new	TTT**AAGCTT**CCCGGCCAGAAGGACACAAAATGAG	HindIII
oEC290	HNE_3409_del2new	ATA**GGATCC**GGATAGTCCGATGGCGGATAAGCGT	BamHI
oEC291	HNE_3409_del3new	ATA**GGATCC**TGGGTCATCCCCGAAGGCTACGAGA	BamHI

Table S6. Oligonucleotides used in this work (continued).

oEC292	HNE_3409_del4new	TATA**GCTAGC**CGCGCTGTATATGCCGCCGGC	NheI
oEC295	HNE_3102_del1new	TTT**AAGCTT**GGGCCGCCACACAAACCTCGTCAGC	HindIII
oEC296	HNE_3102_del4new	TATA**GCTAGC**GCAGGCGCTCATCGAGGCTGACC	NheI
oEC313	HNE_0008_del1	TTT**AAGCTT**AGGATTTCGCCGCCAACATGTC	HindIII
oEC314	HNE_0008_del2	TTT**GGATCC**AAGGGCGGAAAACAGGAGAGCA	BamHI
oEC315	HNE_0008_del3	TTT**GGATCC**GTCGCCGCGCGCACGCCTGCGCC	BamHI
oEC316	HNE_0008_del4	TATAGCTAGCCAAGGACACGGCAGACGAGTAC	NheI
oEC326	HNE_1815_del1	TTT**AAGCTT**GAACAGTATGCGATGGAAAAT	HindIII
oEC327	HNE_1815_del2	TTT**GGATCC**CCGGCCAACAAAAGTCGAACG	BamHI
oEC328	HNE_1815_del3	TTT**GGATCC**GGCACGGCGGACGGCCGGATC	BamHI
oEC329	HNE_1815_del4	TATA**GCTAGC**CGTCATCTTGGCGAGCCGGGC	NheI
oEC331	1815_del_check_rev	GAAGATTCCAGATGCCCCGTC	-
oEC338	HNE_0929_del1	TTT**GGATCC**CGCAAGCGTCAGCAGCAACAG	BamHI
oEC339	HNE_0929_del2	CGC**GGTACC**AAAGAGCAGAAGAAACAGGGT	KpnI
oEC340	HNE_0929_del3	CGC**GGTACC**GTCCCGGAAGGAACCTTCCTG	KpnI
oEC341	HNE_0929_del4	TATA**GCTAGC**CCGGCCTGCATATCCTCGTTG	NheI
oSE20	FtsI-Fwd	AT**GGTACCATG**AGCGAAGCCTCCCGGGATC	KpnI
oSE21	FtsI-Rev	AT**GAGCTC**TCAAAGTTCGCTCCTGTTCTGGG	SacI
oSE88	FtsIflankingup-fwd	AATTAA**AAGCTT**GCAGAGCGCATATTGAAGCCCGGC	HindIII
oSE89	FtsIflankingup-rev	CTTGCTCACCATCTGGGCGCCTCCGCAGG	-
oSE90	VenusftsI'-fwd	GGCGCCCAGATGGTGAGCAAGGGCGAGGAGCTG	-
oSE91	VenusftsI'-rev	AT**GGATCC**AGGCGTATCGCTGGCGCGGTTGG	BamHI
oSR01	HNE_0402_del1	TAGT**AAGCTT**GCGATCGAGACGGATGGATTT	HindIII
oSR02	HNE_0402_del2	TAT**GAATTC**CGCGCCAGAGAGAAAGCGGGG	EcoRI
oSR03	HNE_0402_del3	TAT**GAATTC**GCCAATCTGGTGCGCATCGCC	EcoRI
oSR04	HNE_0402_del4	TATA**GCTAGC**TATCTCCGCTTCAGGGTTTCC	NheI
oSR05	HNE_0445_del1	TAGT**AAGCTT**AACCTGCAGAACAACTAGACG	HindIII
oSR06	HNE_0445_del2	TAT**GAATTC**CAGCATGAACAGACGGCGCAC	EcoRI
oSR07	HNE_0445_del3	TAT**GAATTC**GTCGATCTCAACCGGGCAGCA	EcoRI
oSR08	HNE_0445_del4	TATA**GCTAGC**GGCCATCCATGACTGACAGGT	NheI
oSR09	HNE_1025_del1	TAGT**AAGCTT**TCCAGCAGCACATCAGCGTAG	HindIII
oSR10	HNE_1025_del2	TAT**GAATTC**ATGGACTACACCCGCCGTCAG	EcoRI
oSR11	HNE_1025_del3	TAT**GAATTC**GCGAAACGCACGAGACCAGACT	EcoRI
oSR12	HNE_1025_del4	TATA**GCTAGC**GCGTAGCCCGACTGGATCTGG	NheI
oSR31	HNE_2934_int_rev2	TAGT**AAGCTT**ACCGCGTCGAGGCCATCTGCA	HindIII
oSR32	HNE_2934_int_for3	GAGCCATCAGGGCAGACGACATGGTGAGCAAGGGCGAGGA	-
oSR33	HNE_2934_int_rev4	TCCTCGCCCTTGCTCACCATGTCGTCTGCCCTGATGGCTC	-
oSR70	HNE_3409_int_for1	TAGT**AAGCTT**CTCGCCCTTCTGCCCGGTAAA	HindIII
oSR71	HNE_3409_int_for3	TGGACGAGCTGTACAAGTAAAACAAAAAGGGCGGCTCTCG	-
oSR72	HNE_3409_int_rev4	CGAGAGCCGCCCTTTTTGTTTTACTTGTACAGCTCGTCCA	-
oSR73	HNE_1814_int_for1	TAGT**AAGCTT**CGTCGGCTGTGCGCGATGGCGTGC	HindIII
oSR74	HNE_1814_int_rev2	TATA**GCTAGC**TGGTCTGCGCTCGAACGTGTC	NheI
oSR75	HNE_1814_int_for3	TGGACGAGCTGTACAAGTAAGAGGGTGTTCGCACGTTTAT	-
oSR76	HNE_1814_int_rev4	ATAAACGTGCGAACACCCTCTTACTTGTACAGCTCGTCCA	-
oSR81	HNE_3349_int_for1	TAGT**AAGCTT**CCGCAGGGGTTTGACTTCAGC	HindIII
oSR82	HNE_3349_int_rev2	TATA**GCTAGC**CCCTTCCTGGTCTATGCTGTG	NheI
oSR83	HNE_3349_int_for3	TGGACGAGCTGTACAAGTAAGCGCGTATCCGGGGCGGCGC	-
oSR84	HNE_3349_int_rev4	GCGCCGCCCCGGATACGCGCTTACTTGTACAGCTCGTCCA	-
oSR89	HNE_0674_del1	TAGT**AAGCTT**CCTCTTCAGCCGCTTCCTTCA	HindIII
oSR90	HNE_0674_del2	AA**GGATCC**CAGCTCGGCGGTATGAGACGG	BamHI
oSR91	HNE_0674_del3	AA**GGATCC**TGCTCGAACTTGGCTTCCTGA	BamHI
oSR92	HNE_0674_del4	TATA**GCTAGC**AAAGGGTGTGGCCGCCGTCAG	NheI
oSS271	HNE_0674-rev	AT**GGTACC**TTGGGACGCGAGGCGGAGATCCTG	KpnI

[1] Restriction sites are indicated in boldface.

SUPPLEMENTAL REFERENCES

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