1 Bsub_NahA	1 M-TYLORVINCVLQIDDKVLLLQKPRRGWWVAPGGKMESGESVRDSVIREYREETGIYIINPQLKGVFIFIIKDGDHIVSEWMMFT 85
2 Ban_YvcI(BA5385)	1MORVINCVLIRDNEVLLLOKPRRNWWVAPGGKMERSEIVRDSVVREYREETGIYLKNPALKGVFIFVIQEGDKVVSEWMMFS 82
3 Bhalo_YvcI (BH3570)	1M <mark>QRVTNC</mark> IVVDHDQ <mark>VLLLQKPRRGWWVAPGGKMP</mark> AGESILETVKREYWEETGITVKNPELKGIFSMVIFDEGKI <mark>VSEWM</mark> LFT 82
4 TTherm_Ndx8	1 MRREILVAAAIILLDSRGR <mark>VLL</mark> VGNDWGRRG <mark>R</mark> VRYTL <mark>PGG</mark> TV <mark>P</mark> GGTAVEALV <mark>RE</mark> VREETGLRVRSVEHLA-YVIQVEDRRKN-ERTLAMA 88
5 EcMutT	1 M-KKLOIAVGIIRNENNEIFITRRAADAHMANKLEF <mark>PGGK</mark> IEM <mark>GE</mark> TPEQA <mark>VVRE</mark> LQEEV <mark>GI</mark> TPQHFS <mark>E</mark> FEKLEYEFPDRHITLWFULVER 89
6 EcNudG	1 MKMIE <mark>W</mark> VAAIIERDGKI <mark>MA</mark> GREAQSDQAGL <mark>W</mark> EFA <mark>GGKVE</mark> PD <mark>E</mark> SQRQALV <mark>RE</mark> IR <mark>SE</mark> L <mark>SI</mark> EATVGEYVASHQREVSGRIIHLHAWHVPD 88
	NuDiX Box
1 Bsub NahA	86 EVADSYTSONVSESEECK-LOWHDVNDIONLPMAPGDGHILDFMMKGQGLLHGTFTTTPEFELLSYELDFOHIK 158
1 Bsub_NahA 2 Ban_YvcI (BA5385)	86 FVADSYTGONVSESEEGK-LOWHDVNDIONLPMAPGDGHILDFMMKGQGLLHGTFTTTPEFELLSYRLDPQHIK 158 83 FLATDFAGENKLESEEGI-IGWHTFDKIDDLAMAPGDYH <mark>I</mark> IDYLIKGNGIIYGTFV <mark>TTPDFELLSYRL</mark> DPS 152
1 Bsub_NahA 2 Ban_YvcI (BA5385) 3 Bhalo_YvcI (BH3570)	86 FVADSYTGONVSESEEGK-LOWHDVNDIONLPMAPGDGHILDFMMKGOGLLHGTFTTTPEFELLSYRLDPQHIK 158 83 FLATDFAGENKLESEEGI-IGWHTFDKIDDLAMAPGDYHIIDYLIKGNGIIYGTFVTPDFELLSYRLDPS 152 83 FKATEHEGEMLKOSPEGK-LEWKKKDEVLELPMAAGDKWIFKHVLHSDRLLYGTFHTPDFELLSYRLDPEPQMKKGV 159
1 Bsub_NahA 2 Ban_YvcI (BA5385) 3 Bhalo_YvcI (BH3570) 4 TTherm_Ndx8	 86 FVADSYTGONVSESEEGK-LOWHDVNDIONLPMAPGDGHILDFMMKGQGLLHGTFTTTPEFELLSYRLDPQHIK 158 83 FLATDFAGENKLESEEGI-IGWHTFDKIDDLAMAPGDYHIIDYLIKGNGIIYGTFVTPDFELLSYRLDPS 152 83 FKATEHEGEMLKOSPEGK-LEWKKKDEVLELPMAAGDKWIFKHVLHSDRLLYGTFHYTPDFELLSYRLDPEPQMKKGV 159 89 FRAT-YEGLLNPRDPDGHIVEARFFTLEEVEERLKGHLPLLEPLRA
1 Bsub_NahA 2 Ban_YvcI(BA5385) 3 Bhalo_YvcI(BH3570) 4 TTherm_Ndx8 5 EcMutT	86 FVADSYTGQNVSESEEGK-LQWHDVNDIQNLPMAPGDGHILDFMMKGQGLLHGTFTTTPEFELLSYRLDPQHIK 158 83 FLATDFAGENKLESEEGI-IGWHTFDKIDDLAMAPGDYHIIDYLIKGNGIIYGTFVTPDFELLSYRLDPSDFS 152 83 FKATEHEGEMLKQSPEGK-LEWKKKDEVLELPMAAGDKWIFKHVLHSDRLLYGTFHYTPDFELLSYRLDPSDFS 159 89 FRAT-YEGLLNPRDPDGHIVEARFFTLEEVEERLKGHLPLLEPLRA

Figure S1. Protein sequence alignment of NahA (YvcI) homologs in different species. Multiple sequence alignment of YvcI homologs from *B. subtilis* (Bsub), *B. anthracis* (Ban), *B. halodurans* (Bhalo), *Thermus thermophilus* (TTherm) and *E. coli* (Ec). Alignment was obtained with MAFFT. Red shading indicates identical residues among *Bacillus* and other species, and orange shading indicates identical residues only in *Bacillus* species. The *Bacillus halodurans* YvcI structure (PDB ID: 3FK9) shows its conserved structure as a NuDiX hydrolase. Red dots label the key residues for catalytic activity. Numbers on the sides of the sequences indicate the residue numbers in the corresponding proteins.

Fig. S2



Figure S2. Cell lysate containing Yvcl, but not purified Yvcl, binds (p)ppGpp.

DRaCALA of cell lysate containing His-MBP-tagged *B. subtilis* Yvcl (NahA) and purified His-MBP-tagged *B. subtilis* Yvcl (NahA) with 5'-α-³²P-labeled (p)ppGpp.







a) TLC analysis of pppGpp and ppGpp hydrolysis by NahA over time. The same assay was also performed using GTP. **b)** TLC analysis of ppGpp hydrolysis by Rel and NahA over time. Substrate and product identities are labeled on the left side of the figure. **c)** pppGpp and 8-oxo-GTP hydrolysis reaction, separated by TLC and visualized by UV. 1 mM pppGpp and 8-oxo-GTP were incubated with 200 nM *B. subtilis* NahA at 37°C. Samples were collected at time points denoted in the figure and analyzed by TLC. TLC plate was imaged with UV at wavelength of 254 nm.

Fig. S4

а



Figure S4. nahA complementation rescues pGpp production in vivo.

a) Intensity profile of pGpp from LC-MS measurement of mid log phase wild type, $\Delta nahA$ and nahA complementation strain ($\Delta nahA$ IPTG-nahA). x-axis: retention time in LC-MS; y-axis: ion counts. For each strain, a representative curve was shown. Dashed lines are the shoulders of the GTP signal peak. b) Normalized ion count (ion count per OD_{600nm} per unit volume of the culture) of corresponding pGpp levels, obtained as the average of 3 biological replicates in a). Error bars represent standard error of the mean.

Fig. S5



Figure S5. *nahA* mutant displayed higher (p)ppGpp and lower pGpp *in vivo* in *B. subtilis* in a time course of amino acid starvation.

a-b) pppGpp (a) and ppGpp (b) levels in wild type and $\Delta nahA B$. subtilis cells treated with the nonfunctional arginine analog RHX (arginine hydroxamate). Nucleotide levels were assayed via TLC and are normalized to the ATP level at time zero (ATP_{t=0}). Error bars represent standard errors of mean of three independent experiments. **c-e)** LC-MS analyses of pppGpp (c), ppGpp (d) and pGpp (e) of wild type and $\Delta nahA$ in a time course of RHX treatment. Normalized ion count is ion count per OD_{600nm} per unit volume of the culture. The average shown in figure represents two biological replicates.

Fig. S6 b а С Growth recovery from nutrient downshift Protein translation **Competition Assay** with the presence of guanosine Rate of [³⁵S]-met incorporation (%) 100 100 2 Percentage in total CFU (%) - WT 50 75 0 log2(OD_{600nm}) **_** ∆nahA -2 WТ 25 50 ∆nahA WΤ -4 12.5 25 ∆nahA ∆nahA IPTG-nahA -6 6.25 0 ò 2 ò 100 200 300 400 500 20 . 40 60 6 0 4 Time after RHX treatment (min) Time (day) Time (min)

Figure S6. Phenotypical effects of *nahA* deletion.

a) Protein translation rate of wild type and $\Delta nahA$ cells in a time course of arginine hydroxamate (RHX) treatment. Translation rate was measured by a two minutes pulse of [³⁵S]-methionine incorporation into TCA-precipitable fraction. Error bars represent standard errors of the mean from three replicates. Asterisks indicate statistical significance of differences (* *P*≤0.05, two-tailed two-sample equal-variance Student's *t* test). **b)** Percentage of $\Delta nahA$ and wild type strains obtained from a 7-day serial-dilution competition assay. Error bars represent standard errors of the mean from 3 repeats. **c)** Log phase cultures of wild type, $\Delta nahA$ and *nahA* complementation ($\Delta nahA$ IPTG-*nahA*) strains in rich media with 0.5 mM guanosine were firstly downshifted in minimum media without amino acids for 10 min, and then diluted in rich media with 0.5 mM guanosine for outgrowth. Error bars in represent standard errors from 9 replicates.

	pppGpp	ppGpp	pGpp
HflX	5 ± 1 µM	3.0 ± 0.6 μM	ND
Obg	48 ± 7 μM	6 ± 2 µM	ND
EF-G	296 ± 50 µM	60 ± 8 µM	ND

Supplementary Table 1. $K_d \pm SD$ of GTPases to pppGpp, ppGpp and pGpp. ND: not detected.