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A new thiamin salvage pathway

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The physiological function for thiaminase II, a thiamin-degrading enzyme, has eluded investigators for more than 50 years. Here, we demonstrate that this enzyme is involved in the regeneration of the thiamin pyrimidine rather than in thiamin degradation, and we identify a new pathway involved in the salvage of base-degraded forms of thiamin. This pathway is widely distributed among bacteria, archaea and eukaryotes. In this pathway, thiamin hydrolysis products such as *N*-formyl-4-amino-5-aminomethyl-2-methylpyrimidine (formylamino-pyrimidine; 15) are transported into the cell using the ThiXYZ transport system, deformylated by the *ylmB*-encoded amidohydrolase and hydrolyzed to 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP; 6)—an intermediate on the *de novo* thiamin biosynthetic pathway. To our knowledge this is the first example of a thiamin salvage pathway involving thiamin analogs generated by degradation of one of the heterocyclic rings of the cofactor.

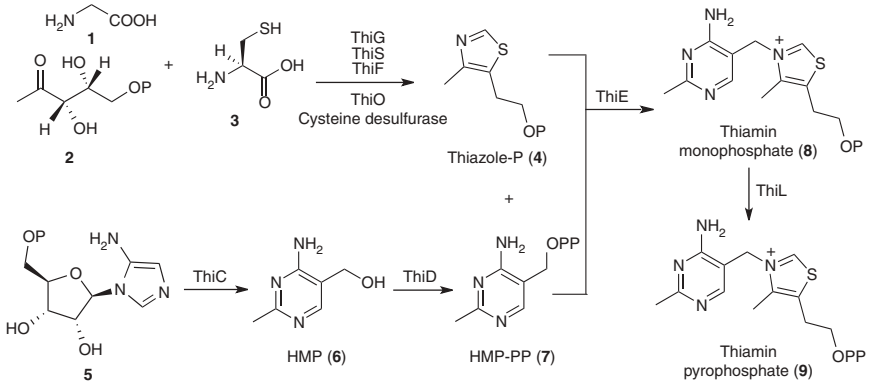
Thiamin is an essential cofactor in all living systems. It is biosynthesized *de novo* in microorganisms and plants via a complex pathway, but it is not synthesized in humans and is therefore an essential nutrient. The biosynthesis of thiamin by microorganisms has been studied extensively and most of the genes involved have been characterized (**Scheme 1**)[1,2]. In *Bacillus subtilis*, HMP-PP (7) is produced by rearrangement of aminoimidazole ribonucleotide (AIR; 5)[3] followed by phosphorylation[4], and the thiazole phosphate (4) is formed by an oxidative condensation of glycine (1), 1-deoxy-D-xylulose-5-phosphate (DXP; 2) and cysteine (3)[5]. Thiamin phosphate (8) is then formed by the coupling of the pyrimidine and the thiazole heterocycles[6]. A final phosphorylation gives thiamin pyrophosphate[7] (9), the biologically active form of the cofactor.

TenA was recently identified as a bacterial thiaminase II, an enzyme that catalyzes the hydrolysis of thiamin (10) to give HMP and hydroxyethylthiazole (11) (**Scheme 2**)[8]. In many bacteria, the *tenA* gene clusters on the chromosome with or is fused to other thiamin biosynthetic genes (**Supplementary Fig. 1** online)[9]. This chromosomal clustering is not consistent with a degradative function for this enzyme; therefore, though the structure and previously identified activity of TenA strongly suggested that it catalyzes a pyrimidine substitution reaction[8], thiamin is unlikely to be the physiological substrate for this enzyme.

Analysis of the genes that cluster on the chromosome with *tenA* in sequenced bacteria provided important clues as to the identity of the TenA substrate (**Supplementary Fig. 1**). In *B. subtilis*, *tenA* is clustered on the chromosome with a set of genes involved in the biosynthesis of the thiazole moiety of thiamin (*thiO*, *thiS*, *thiG*, *thiF*) and HMP pyrophosphorylation (*thiD*), whereas in *Bacillus halodurans tenA* clusters with a putative amidohydrolase (*ylmB*) and an ABC transporter (*thiX*, *thiY*, *thiZ*)[10]. In *Bacillus cereus*, *tenA* clusters with *thiX*, *thiY* and *thiZ* and *thiE*, *thiO*, *thiS*, *thiG*, *thiF* and *thiD*. Each of these clusters also contains an upstream TPP riboswitch, which suggests thiamin regulation[11,12]. The sequence similarity of ThiY to the HMP synthase (Thi5) in *Saccharomyces cerevisiae* suggests that ThiXYZ is involved in the transport of HMP analogs[10]. Given that *B. halodurans* grows in basic soil[13,14] (pH > 10), it seemed likely that these pyrimidine analogs could be generated by base-catalyzed degradation of the thiamin thiazole in soil (**Fig. 1a**)[15,16]. We therefore proposed that YlmB, TenA and ThiXYZ are involved in the salvage of HMP from base-degraded forms of thiamin formed in soil.

This paper describes the experimental validation of this hypothesis and the identification of the first example of a thiamin salvage pathway involving a heterocycle-degraded form of the cofactor.

Though the high soil pH at which *B. halodurans* grows was an important clue to the function of TenA, the proposed thiamin



Scheme 1 The main pathway for thiamin pyrophosphate biosynthesis in bacteria.

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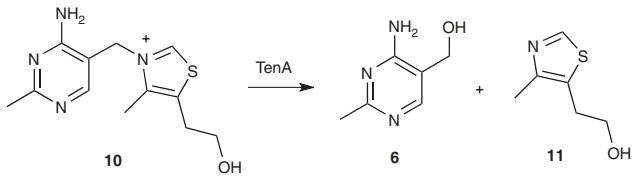


Scheme 2 The previously identified TenA-catalyzed thiamin degradation reaction.

degradation chemistry must also occur under neutral conditions because *B. subtilis* and *B. cereus* both grow in neutral soil. We therefore focused our attention on thiamin degradation under the milder reaction conditions in neutral soil to simplify the decomposition reactions mixtures. Even under these conditions, HPLC analysis of a reaction mixture generated by treating thiamin with sterilized soil (pH 7, 15 d, 25 °C) revealed the presence of a large number of thiamin degradation products relative to a soil-free control that showed only low levels of decomposition after 6 months (**Supplementary Fig. 2** online). This thiamin decomposition reaction is presumably catalyzed by metal-ion-bound hydroxide on the clay surface, as has been described previously for other clay-catalyzed hydrolysis reactions[17].

A solution of soil-degraded thiamin was treated with the recombinant purified proteins YlmB and TenA at room temperature for 3 h. HPLC analysis of the resulting reaction mixture demonstrated that in addition to the expected TenA-catalyzed thiamin hydrolysis, a component eluting at 15 min (X_{15}) disappeared from the reaction mixture (**Supplementary Fig. 3** online). In the absence of YlmB, this compound was unaltered under these reaction conditions.

To determine whether any additional YlmB or TenA substrates were buried in the complex mixture of compounds eluting between 18 and 25 min, HPLC fractions containing these compounds were collected, treated with a mixture of TenA and YlmB and reanalyzed by HPLC (**Supplementary Fig. 4** online). None of the components of this mixture were converted to HMP, which demonstrates that these compounds are not relevant to the proposed HMP salvage pathway.

The crude thiamin decomposition mixture was greatly enriched in the YlmB substrate (X_{15}) by chromatography on silica gel to remove the positively charged unreacted thiamin from the neutral degradation products. The resulting mixture was again treated with YlmB and TenA. HPLC analysis of this reaction mixture demonstrated that a combination of YlmB and TenA catalyzes the conversion of X_{15} to HMP (**Fig. 1b**).

X_{15} was further purified by HPLC. Its structure was determined to be *N*-formyl-4-amino-5-aminomethyl-2-methylpyrimidine (**15**, **Fig. 1a**) by mass spectrometry and NMR analysis. A mechanistic proposal for the formation of this product is outlined in **Figure 1a** (ref. 16). In this mechanism, water addition to the thiazolium heterocycle of **10** gives **12**, which then undergoes ring opening to give **13**. Tautomerization to **14** followed by hydrolysis gives the formylaminopyrimidine (**15**).

Formylaminopyrimidine (**15**) was synthesized by formylation of aminopyrimidine (**17**) and demonstrated to be identical to X_{15} by NMR, mass spectrometry and HPLC analysis (**Supplementary Fig. 5** online). YlmB catalyzed the deformylation of **15** to give aminopyrimidine, and TenA catalyzed the hydrolysis of this aminopyrimidine to give hydroxypyrimidine (**6**) (**Fig. 1c**). The hydroxypyrimidine formed by TenA can then be phosphorylated by ThiD and incorporated into the thiamin biosynthetic pathway (**Scheme 1**). Formylaminopyrimidine is not a substrate for TenA and is stable under the reaction conditions. We have also demonstrated that ThiY (the putative substrate-binding component of the ABC transporter

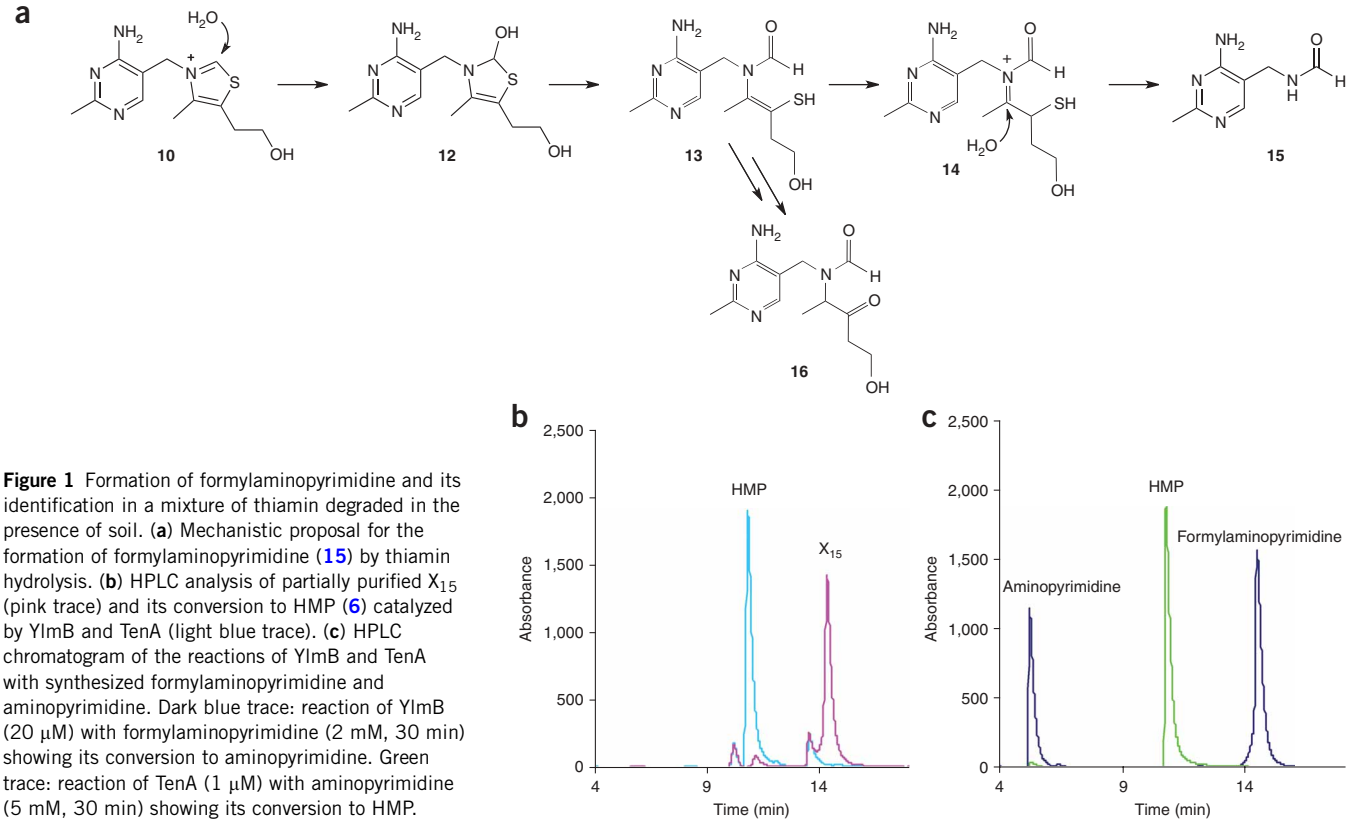
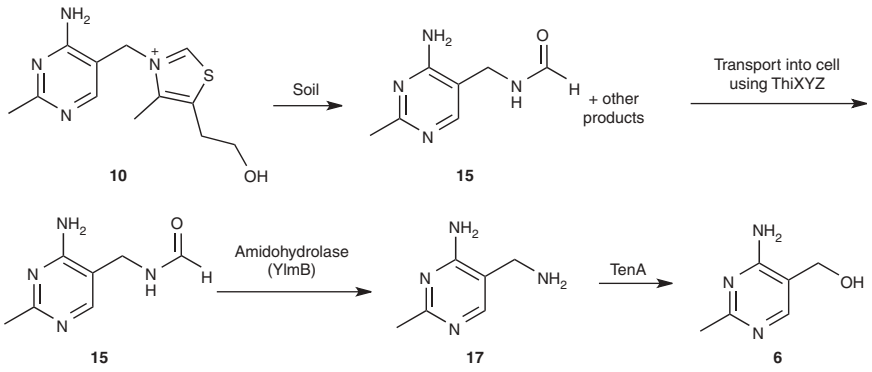


Figure 1 Formation of formylaminopyrimidine and its identification in a mixture of thiamin degraded in the presence of soil. (**a**) Mechanistic proposal for the formation of formylaminopyrimidine (**15**) by thiamin hydrolysis. (**b**) HPLC analysis of partially purified X_{15} (pink trace) and its conversion to HMP (**6**) catalyzed by YlmB and TenA (light blue trace). (**c**) HPLC chromatogram of the reactions of YlmB and TenA with synthesized formylaminopyrimidine and aminopyrimidine. Dark blue trace: reaction of YlmB (20 μM) with formylaminopyrimidine (2 mM, 30 min) showing its conversion to aminopyrimidine. Green trace: reaction of TenA (1 μM) with aminopyrimidine (5 mM, 30 min) showing its conversion to HMP.



Scheme 3 The identified functions for ThiY, YlmB and TenA, which suggest a new salvage pathway for the thiamin pyrimidine (**6**).

ThiXYZ) binds formylaminopyrimidine and probably delivers it to the ThiXYZ transport system. These identified functions suggest the HMP salvage pathway shown in **Scheme 3**.

The kinetic parameters for the YlmB-catalyzed reaction are k_{cat} = 14.0 ± 0.7 min^{-1}, K_m = 5 ± 1.6 μM and k_{cat}/K_m = 2.8 ± 0.97 min^{-1} μM^{-1}, and the kinetic parameters for the TenA-catalyzed reaction are k_{cat} = 22.0 ± 0.48 min^{-1}, K_m = 11.8 ± 1.6 μM and k_{cat}/K_m = 1.9 ± 0.26 min^{-1} μM^{-1}. The YlmB assay was based on formate detection using formate dehydrogenase and monitoring NADH production[18], whereas the TenA assay was based on ammonia detection using α-ketoglutarate/glutamate dehydrogenase and monitoring NADPH consumption[19]. The equilibrium binding constant for the formylaminopyrimidine binding to ThiY was determined to be 200 nM by measuring the change in protein fluorescence upon binding. Thiamin does not bind to ThiY.

To evaluate the *in vivo* relevance of this HMP salvage pathway, we examined the ability of TenA and ThiA (HMP-requiring) mutants in *B. subtilis* to grow on formylaminopyrimidine, aminopyrimidine, and thiamin that was completely base degraded. The results of these experiments are shown in **Table 1** and **Supplementary Figure 6** online. The ThiA mutant is HMP-requiring, as expected, and grows on all of the hydroxypyrimidine sources. The TenA mutant does not require a hydroxypyrimidine source as it is able to biosynthesize it using ThiA. The TenA/ThiA double mutant, however, is hydroxypyrimidine-requiring and is unable to salvage the pyrimidine from formylaminopyrimidine, aminopyrimidine, or base-degraded thiamin.

Although we have not carried out a comprehensive study on the substrate tolerance of TenA or YlmB, it is highly likely that other analogs of thiazole-degraded thiamin are also substrates for this salvage pathway. In our identification of formylaminopyrimidine as a substrate for YlmB, we deliberately chose mild thiamin degradation conditions (soil, pH = 7) to simplify the degradation reaction mixtures and to mimic biological conditions. Under harsher reaction conditions, a larger set of reaction products is generated and it is likely that several additional substrates for YlmB and TenA exist in such mixtures. We have demonstrated that degradation product **16**[20] (**Fig. 1a**) is not a substrate for YlmB or TenA, whereas an analog of **15** with a hydroxyethyl substituent (**19**) on the amide nitrogen is a good substrate (data not shown). This demonstrates that some degree of degradation of the C5 chain of the thiazole is required to generate YlmB substrates.

The previously reported thiaminase II activity of TenA is unlikely to be physiologically relevant because TenA can be overexpressed in *Escherichia coli* at a high level without inducing toxicity or a thiamin requirement. This is most likely due to the substrate selectivity of TenA, which catalyzes the hydrolysis of thiamin but not thiamin phosphate or pyrophosphate, the only forms of biosynthesized thiamin in the cell. In addition, though the TenA-catalyzed thiamin hydrolysis does not follow Michaelis-Menten kinetics, it was possible to determine, using a competition assay, that TenA catalyzes the hydrolysis of aminopyrimidine 100 times faster than it catalyzes the hydrolysis of thiamin, thereby further adding to the selectivity of TenA for thiamin degradation products.

The three previously identified pathways for thiamin salvage involve phosphorylation of the biosynthetic intermediates HMP, thiamin and hydroxyethylthiazole[21–24]. These pathways were relatively easy to find as an extension of the biosynthetic studies. The identification of a salvage pathway involving products resulting from the degradation of one of the thiamin heterocyclic rings suggests that additional salvage pathways involving other thiamin decomposition products are likely to exist.

The identification of the physiological function of thiaminase I and II has been a long-standing unsolved problem. Although we do not yet clearly understand the physiological function of thiaminase I, the experiments described in this paper suggest that pyrimidine salvage rather than thiamin degradation is the physiological function of thiaminase II. The wide distribution of TenA in bacteria, archaea and eukaryotes suggests the presence of this salvage pathway in all three kingdoms of life. YlmB is less widely distributed and frequently not present in organisms containing TenA, which suggests that the deformylation of **15** can be catalyzed by a variety of amidohydrolases. The wide distribution of TenA also suggests that destruction of the thiamin thiazole is a commonly occurring thiamin degradation reaction and that this new salvage pathway is widespread and not restricted to soil-growing bacteria.

METHODS

Soil-catalyzed thiamin degradation. A solution of thiamin (100 ml, 100 mM, pH 7 phosphate buffer) containing 10 g of soil was vigorously stirred at room temperature for 15 d and filtered. The resulting solution was used as a substrate for YlmB and TenA. The soil was obtained from the lawn behind S.T. Olin laboratory at Cornell University in Ithaca, New York, USA and sterilized by autoclaving at 120 °C for 20 min before use.

Table 1 Complementation studies on ThiA and TenA mutants in *B. subtilis*

B. subtilis strain/mutant	Thiamin	HMP	Aminopyrimidine	Formylaminopyrimidine	Base-degraded thiamin	Nothing
ThiA	+	+	+	+	+	–
TenA	+	+	+	+	+	+
ThiA TenA	+	+	–	–	–	–
WT	+	+	+	+	+	+

Purification of X_{15} was carried out by slowly pouring 4 ml of this reaction mixture onto a one-inch layer of silica previously equilibrated in 90% chloroform, 10% methanol in a sintered glass funnel. The silica was then washed with 1.5 l of 90% chloroform, 10% methanol, the solvent was removed and the resulting sample of crude X_{15} was dissolved in 3 ml of water. A 250 µl aliquot of this sample was further purified by HPLC as described below. The fractions containing X_{15} in phosphate buffer were collected, the solvent was removed and the sample was dissolved in D_2O. An NMR spectrum was obtained on a Bruker 300 MHz instrument, δ 2.33 (s, 3H), 4.21 (s, 2H), 7.91 (s, 1H), 8.12 (s, 1H). The dried sample was extracted from the phosphate salts with methanol and analyzed by ESI mass spectrometry (Esquire mass spectrometer from Bruker with an ion-trap mass analyzer) to give a single ion with an *m/z* [M^+] ratio of 167.

Synthesis of formylaminopyrimidine (15). A suspension of aminopyrimidine (17) (276 mg, 2.0 mmol) and 1H-benzotriazole-1-carboxyaldehyde (18) (Sigma) (294 mg, 2.0 mmol) in acetonitrile (30 ml) was stirred at room temperature for 1 h. Filtration and washing with acetonitrile (15 ml) gave pure product as a white solid (240 mg, 72%). ^{1}H NMR (300 MHz, D_2O): δ 2.33 (s, 3H, CH_3), 4.21 (s, 2H, CH_2), 7.92 (s, 1H, CH), 8.12 (s, 1H, NCHO). ^{13}C NMR (300 MHz, D_2O): δ 23.71, 35.55, 110.68, 153.85, 161.62, 164.74, 166.59. ESI mass spectrometry (Esquire mass spectrometer from Bruker with an ion-trap mass analyzer) gave a single ion with (*m/z*): $[M]^+$ calcd. for formylaminopyrimidine, 167.2; found, 167.4.

Synthesis of *N*-((4-amino-2-methylpyrimidin-5-yl)methyl)-*N*-(2-hydroxyethyl)formamide (19). Ethanolamine (20) (940 mg, 15.4 µmol) was added to a solution of 5-(chloromethyl)-2-methylpyrimidin-4-amine (21)[25] (1.54 mmol) in chloroform (30 ml). The reaction was stirred at room temperature for 12 h. Solvent was removed and chromatography (silica gel, chloroform/methanol 5:1) afforded 2-((4-amino-2-methylpyrimidin-5-yl)methylamino)ethanol (22) as a white solid (180 mg, 62%). ^{1}H NMR (300 MHz, CD_3OD): δ 2.38 (s, 3H, CH_3), δ 2.69 (t, J = 5.4 Hz, 2H, CH_2N), δ 3.65 (t, J = 5.4 Hz, 2H, CH_2O), δ 3.69 (s, 2H, CH_2N), δ 7.90 (s, 1H, CH). ^{13}C NMR (500 MHz, DMSO-d6): δ 25.16, 47.28, 50.61, 60.19, 111.87, 153.56, 162.47, 165.14. ESI mass spectrometry (Esquire mass spectrometer from Bruker with an ion-trap mass analyzer) gave a single ion with (*m/z*): $[M]^+$ calcd. for 22, 183.2; found, 183.4.

A solution of 22 (100 mg, 0.53 mmol) and 18 (100 mg, 0.69 mmol) in acetonitrile (20 ml) was stirred at room temperature for 1 h. Chromatography (silica gel, acetonitrile/methanol 2:1) afforded 19 as a white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 2.38 (s, 3H, CH_3) δ 3.33 (t, J = 5.1Hz, 2H, CH_2), δ 3.65 (t, J = 5.1 Hz, 2H, CH_2), δ 4.43 (s, 2H, CH_2), δ 7.99 (s, 1H, CH), δ 8.15 (s, 1H, NCHO). ^{13}C NMR (500 MHz, DMSO-d6): δ 24.90, 40.92, 50.10, 59.82, 110.30, 156.24, 163.61, 166.64, 168.26. ESI mass spectrometry (Esquire mass spectrometer from Bruker with an ion-trap mass analyzer) gave a single ion with (*m/z*): $[M]^+$ calcd. for 19, 211.2; found, 211.4.

Analysis of the thiamin degradation and the TenA/YlmB reaction mixtures. All HPLC analysis was performed on a C_{18} column (Supelco, Supelcosil LC-18-T 15 cm × 4.6 cm, 3 µm) equilibrated in 100 mM phosphate buffer at pH 6.6 using a flow rate of 1 ml min^{-1}. The elution method used a gradient defined as follows: 0–3 min 100% phosphate buffer (100 mM, pH 6.6); 3–4 min 90% phosphate buffer, 10% water; 4–10 min 60% phosphate buffer, 33% water, 7% methanol; 10–15 min 60% phosphate buffer, 33% water, 7% methanol; 15–20 min 10% phosphate buffer, 30% water, 60% methanol; 20–30 min 60% phosphate buffer, 33% water, 7% methanol; 30–35 min 100% phosphate buffer. Reaction products were detected by measuring absorbance at 254 nm.

Bioinformatics tools. Chromosomal clustering of *tenA* was analyzed using The SEED database (http://theseed.uchicago.edu/FIG/index.cgi).

Overexpression and purification of YlmB. PCR amplification of the *ylmB* gene (GenBank ID 15615241) from *B. halodurans* genomic DNA was performed in the standard way using Pfx Platinum polymerase from Invitrogen. The *ylmB* gene was then cloned into a pET16b vector using Nde1 and Xho1 restriction sites. After cloning, the *ylmB* plasmid was transformed into the BL21(DE3) expression strain of *E. coli*. YlmB was obtained by inoculating a 1-l culture of LB medium, containing 100 mg l^{-1} ampicillin, with a 10-ml starter culture. The cells were grown to an optical density at 600 nm (OD_{600}) of 0.6 at 37 °C, at which point expression was induced with 1 mM IPTG. After induction, the cells were grown for 8 h, pelleted, resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole), lysed by sonication, and then clarified by centrifugation at 39,000*g*. The resulting cell-free extract was passed through a Ni-NTA column (Qiagen), which was then washed with 5 column volumes of lysis buffer followed by 300 ml of wash buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 20 mM imidazole). The pure protein was eluted with elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 250 mM imidazole) and buffer exchanged by gel filtration into 100 mM phosphate buffer pH 7 containing 100 mM NaCl to prevent protein precipitation.

Overexpression and purification of TenA. *tenA* (from *B. subtilis*, GenBank ID 16078230) was cloned into a pDESTF1 plasmid (a Gateway-adapted vector based on the pET system from Novagen containing a His_6 tag) and transformed into the BL21Star (DE3)pRare2 expression strain of *E. coli*. TenA was obtained by inoculating a 1-l culture of LB medium, containing 100 mg l^{-1} ampicillin, with a 10-ml starter culture. The cells were grown to an OD_{600} of 0.6 at 37 °C, at which point expression was induced with 1 mM IPTG. After induction, the cells were grown for 8 h, pelleted, resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole) and lysed by sonication. The resulting extract was clarified by centrifugation at 39,000*g* and passed over a Ni-NTA column (Qiagen), which was then washed with 5 column volumes of lysis buffer followed by 300 ml of wash buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 20 mM imidazole). The pure protein was eluted with elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 250 mM imidazole) and buffer exchanged by dialysis against 100 mM phosphate buffer pH 7. The extinction coefficient of TenA was determined to be 74,280 $M^{-1}cm^{-1}$ using a previously described method[26].

Overexpression and purification of ThiY. PCR amplification of the *thiY* gene (GenBank ID 15615245) from *B. halodurans* genomic DNA was performed in the standard way using Pfx Platinum polymerase from Invitrogen. To increase the solubility of ThiY the N-terminal 20 amino acids were truncated to remove a hydrophobic membrane anchor. The truncated *thiY* gene was then cloned into a pET16b plasmid using Nde1 and Xho1 restriction sites. After cloning, the *thiY* plasmid was transformed into the BL21(DE3) expression strain of *E. coli*. ThiY was obtained by inoculating a 1-l culture of LB medium, containing 100 mg l^{-1} ampicillin, with a 10-ml starter culture. The cells were grown to an OD_{600} of 0.6 at 37 °C, at which point expression was induced with 1 mM IPTG. After induction, the cells were grown for 8 h, pelleted, resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole) and lysed by sonication. The resulting extract was clarified by centrifugation at 39,000*g* and passed over a Ni-NTA column (Qiagen), which was then washed with 5 column volumes of lysis buffer followed by 300 ml of wash buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 20 mM imidazole). The pure protein was eluted with elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 250 mM imidazole) and buffer exchanged by gel filtration into 100 mM phosphate buffer pH 7.

Assay for YlmB-catalyzed hydrolysis of formylaminopyrimidine (15). The well-characterized formate dehydrogenase assay[18] was used to measure the YlmB-catalyzed production of formate from formylaminopyrimidine. Before kinetic analysis, 500 µM EDTA was added to YlmB (1 µM) to remove any metal that may have bound during purification, such as Ni^{+2} from the Ni-NTA resin. After incubation, EDTA was removed by gel filtration. In each assay the concentrations of formate dehydrogenase, NAD^+ and YlmB were held constant and the substrate concentration was varied. The oxidation of formate was not rate limiting in any of the assays. Each reaction contained 1 unit of formate dehydrogenase (a unit is defined as the amount that will oxidize 1.0 µmol of formate to CO_2 per min in the presence of NAD at pH 7.6 at 37 °C), 1 mM NAD^+, 1 µM YlmB, 5 µM Co^{+2}. The reactions were started by the addition of formylaminopyrimidine with the final concentrations varying from 5 µM to 400 µM. The YlmB concentration was determined using the Bradford assay, and NADH production was monitored by measuring the increase in absorbance at 340 nm. Kinetic parameters were obtained by fitting the initial rate values (less than 10% conversion) to the Michaelis-Menton equation at

varying substrate concentrations using the fitting program GraFit (Erithacus Software Ltd.). The parameters are: $k_{cat} = 14.0 \pm 0.7$ min^{-1}, $K_m = 5 \pm 1.6$ μM and $k_{cat}/K_m = 2.8 \pm 0.97$ min^{-1} μM^{-1}.

Assay for TenA-catalyzed hydrolysis of aminopyrimidine (17). The well-characterized glutamate dehydrogenase–based assay[19] was used to measure the production of ammonia from aminopyrimidine catalyzed by TenA. In the kinetic assays the concentrations of NADPH, α-ketoglutarate, glutamate dehydrogenase and EDTA were kept constant, while the aminopyrimidine concentration was varied from 10 μM to 500 μM. Each reaction contained 5 units of glutamate dehydrogenase (unit is defined as the amount of glutamate dehydrogenase that will reduce 1 μmol of α-ketoglutarate to glutamate per min at pH 8.3 at 30 °C), 5 mM α-ketoglutarate, 0.1 mM EDTA, and 250 μM NADPH. The reduction of α-ketoglutarate to glutamate was not rate limiting in any of the assays. All reactions were performed by adding each reaction component to a solution of aminopyrimidine. The reaction was then started by adding TenA (0.5 μM). The TenA concentration was determined from its extinction coefficient, and NADPH consumption was monitored by measuring the decrease in absorbance at 340 nm. Kinetic parameters were obtained by fitting the initial rate values (less than 10% conversion) to the Michaelis-Menton equation at varying substrate concentrations using GraFit. The parameters are: $k_{cat} = 22.0 \pm 0.48$ min^{-1}, $K_m = 11.8 \pm 1.6$ μM and $k_{cat}/K_m = 1.9 \pm 0.26$ min^{-1} μM^{-1}.

Determination of the selectivity of TenA for aminopyrimidine versus thiamin. TenA (10 μM) was incubated with equimolar amounts (5 mM) of thiamin (10) and aminopyrimidine. The reaction was quenched after 2 min by filtration through a membrane with a molecular weight cutoff of 10 kDa to remove the TenA protein. After the quench, 100 μl of the filtrate was analyzed by HPLC. The molar ratio of the products of the hydrolysis of thiamin and aminopyrimidine were determined using standard curves (peak area as a function of moles of compound). The molar ratio of these products was used as a measurement of relative rate. The extent of thiamin hydrolysis was determined from the moles of thiazole (11) produced. The product of the aminopyrimidine hydrolysis was determined by subtracting the moles of thiazole produced from the total moles of hydroxypyrimidine (6) produced (thiamin hydrolysis results in a 1:1 ratio of thiazole to hydroxypyrimidine, **Scheme 2**), to provide the moles of hydroxypyrimidine that were produced exclusively from aminopyrimidine. Hydrolysis of thiamin resulted in 0.0002 ± 0.000008 μmol of product; hydrolysis of aminopyrimidine resulted in 0.02 ± 0.004 μmol of product. From this data, we calculate a relative rate of aminopyrimidine hydrolysis to thiamin hydrolysis of 99 ± 4.

Bacterial sources of YlmB, TenA and ThiY. The characterization of the pyrimidine salvage pathway was performed using YlmB and ThiY cloned from *B. halodurans* and TenA cloned from *B. subtilis* because these enzymes were the ones that overexpressed and purified to give the most soluble protein. The YlmB from *B. subtilis* has been overexpressed and catalyzes the same reaction as YlmB from *B. halodurans*, and the TenA from *B. halodurans* has also been overexpressed and catalyzes the same reaction as TenA from *B. subtilis*. Therefore, the pyrimidine salvage pathway has been experimentally characterized in both *B. subtilis* (in which TenA is not clustered with YlmB) and *B. halodurans* (in which TenA is clustered with YlmB).

Determination of the formylaminopyrimidine/ThiY binding constant. The dissociation constant (K_d) for the binding of formylaminopyrimidine (15) to ThiY was determined by fluorescence titration, in which the decrease in protein fluorescence was measured as a function of substrate concentration. Small aliquots (0.75 μl, 186 μM) of substrate were added to a ThiY solution (3 μM) and the protein fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian). The protein was excited at a wavelength of 280 nm and emission was detected at 340 nm, with slit widths of 5 mm for both excitation and emission. The K_d value was obtained by fitting the fluorescence data by nonlinear regression to the following quadratic equation:

$$F_{obs} = F_o + \Delta F \cdot \frac{P_o + L_o + K_d - \sqrt{(P_o + L_o + K_d)^2 - 4 \cdot P_o \cdot L_o}}{2 \cdot P_o}$$

where F_o is the y intercept, ΔF is the overall change in fluorescence, P_o is the protein concentration and L_o is the ligand concentration added[27]. All nonlinear regression was performed in the program GraFit 5. The K_d for the binding of formylaminopyrimidine to ThiY is 200 nM.

Construction of *B. subtilis* mutants. The *thiA* auxotroph THI2 strain was constructed by transforming wild-type strain 1A747 (Bacillus Genetic Stock Center) with a low noncongressional concentration of strain 1A603 (containing the *thiA::Tn917* mutation) chromosomal DNA, with selection on rich medium containing erythromycin (50 mg ml^{-1})[28]. Strain TH140, which contains a deletion of the *tenA* gene, was constructed as follows: long flanking homology PCR[29] was used to generate a deletion mutation in the coding region of the *tenA* open reading frame, in which a 530-base-pair-long nucleotide region of *tenA* was replaced with the chloramphenicol acetyltransferase (encoded by *cat4*) resistance cassette[28]. To do this, two PCR fragment 'arms' were first created: 0.2 μl of a 100 μM solution of primers P1tenA and P2tenA-cat or primers P3tenA-cat and P4tenA (**Supplementary Table 1** online) were added to 0.1 μg 1A747 chromosomal DNA in a 50-μl reaction volume containing 1 μl of 40 mM dNTPs, 5 μl of 10× buffer and 0.75 μl of PCR enzyme (Taq and Tgo), as described by the manufacturer (Expand high-fidelity PCR system, Roche Applied Science). The PCR reaction was performed for 30 cycles using an annealing temperature of 55.7 °C and an elongation time of 45 s. The resulting fragments, called F1 and F2, respectively, were purified and next used as primers in a second round of PCR. F1 and F2 fragments were diluted 50-fold and 1 μl of each was added to 0.1 μg of chromosomal DNA from strain TH12 in a 50-μl reaction volume to amplify the *cat4* cassette. In the first 10 cycles, an annealing temperature of 63 °C and an elongation time of 3 min was used. In the next 20 cycles, the elongation time was extended by 20 s after each cycle. The resulting products were then used in a third round of PCR as a template. The PCR products were diluted 50-fold and 1 μl was combined with 0.2 μl of a 100 μM solution of primer P1ten A and P4tenA in a 50-μl reaction volume containing dNTPs, buffer and enzyme as described above. The PCR reaction parameters were identical to those used in the second-round PCR. The finished PCR fragments were next transformed into the wild-type strain 1A747, selecting for 5 mg ml^{-1} chloramphenicol resistance on TBAB (Difco) medium (Cm^r). A single Cm^r colony deleted for *tenA* was isolated and named TH140 (*DtenA::cat*). The presence of the *cat* cassette was confirmed by diagnostic PCR using P1tenA and P4ten, using standard reaction conditions. Finally, both *thiA* and *tenA* mutations were combined by moving the *tenA::cat* deletion into the THI2 background at noncongressional DNA concentrations. The resulting recombinant strain was named TH141.

Complementation growth conditions. Complementation studies were performed using minimal medium (Spizizen salts, 0.04% tryptophan, 0.04% sodium glutamate and 1% glucose) supplemented with chloramphenicol (5 μg ml^{-1}), erythromycin (1 μg ml^{-1}) or both depending on the antibiotic resistance of the *B. subtilis* mutant. Thiamin and related supplements (**Table 1**) were at a final concentration of 5 μM. Base-degraded thiamin was prepared by reacting thiamin (1 M) with 5 M NaOH for 9 d followed by neutralization with HCl and chromatography on a plug of silica equilibrated with 90% chloroform, 10% methanol as described above. This sample was thiamin-free by HPLC analysis.

Accession codes. GenBank: the following sequences were deposited as part of previous studies: the *ylmB* gene from *B. halodurans* genomic DNA (GenBank ID 15615241), *tenA* from *B. subtilis* (GenBank ID 16078230), and the *thiY* gene from *B. halodurans* genomic DNA (GenBank ID 15615245).

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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