Published in final edited form as:

ACS Chem Biol. 2019 April 19; 14(4): 742–750. doi:10.1021/acscchembio.9b00030.

Molecular basis of *Bacillus subtilis* ATCC6633 self-resistance to the phosphono-oligopeptide antibiotic rhizocticin

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Abstract

Rhizocticins are phosphono-oligopeptide antibiotics that contain a toxic C-terminal (*Z*)-*L*-2-amino-5-phosphono-3-pentenoic acid (APPA) moiety. APPA is an irreversible inhibitor of threonine synthase (ThrC), a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the conversion of *O*-phospho-*L*-homoserine to *L*-threonine. ThrCs are essential for the viability of bacteria, plants, and fungi and are a target for antibiotic development, as *de novo* threonine biosynthetic pathway is not found in humans. Given the ability of APPA to interfere in threonine metabolism, it is unclear how the producing strain *B. subtilis* ATCC6633 circumvents APPA toxicity. Notably, in addition to the housekeeping APPA-sensitive ThrC (*Bs*ThrC), *B. subtilis* encodes a second threonine synthase (RhiB) encoded within the rhizocticin biosynthetic gene cluster. Kinetic and spectroscopic analyses show that PLP-dependent RhiB is an authentic threonine synthase, converting *O*-phospho-*L*-homoserine to threonine with a catalytic efficiency comparable to *Bs*ThrC. To understand the structural basis of inhibition, we determined the crystal structure of APPA bound to the housekeeping *Bs*ThrC, revealing a covalent complex between the inhibitor and PLP. Structure-based sequence analyses reveal the molecular determinants within the RhiB active site responsible for rendering this ThrC homolog resistant to APPA. Together, this work establishes the self-resistance mechanism utilized by *B. subtilis* ATCC6633 against APPA exemplifying one of many ways by which bacteria can overcome phosphonate toxicity.

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INTRODUCTION

Phosphonates are natural products characterized by the presence of an inert C-P bond in place of the more common O-P bond found in phosphoric acids.¹ As structural analogs of numerous phosphate ester and carboxylic acid intermediates found in metabolic processes, phosphonates act as inhibitors of the corresponding enzymes. For instance, the unusual non-proteinogenic amino acid (*Z*)-*L*-2-amino-5-phosphono-3-pentenoic acid (APPA) inhibits threonine synthases (ThrC) irreversibly by mimicking the physiological substrate *O*-phospho-*L*-homoserine (PHSer) (Figure 1a).^{2, 3} ThrC is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that utilizes PHSer as a substrate in the last step in threonine biosynthesis (Figure 1a).^{4, 5}

APPA is the pharmacophore present at the C-terminus of the phosphono- oligopeptide antifungal rhizocticin,⁶ produced by the Gram-positive bacterium *Bacillus subtilis* ATCC6633 (Figure 1b).⁷ Notably, APPA is also found as the active warhead of plumbemycins, which are antibacterial tripeptides produced by *Streptomyces plumbus*.^{8, 9} Although both phosphono-oligopeptides contain a C-terminal APPA residue, they contain different amino acids at their N-terminus. This difference is believed to be responsible for defining the spectrum of target organisms affected by the two natural products. Thus, due to differences in the specificities of oligopeptide transporters, rhizocticin has antifungal, but not antibacterial, activity, while plumbemycin has the opposite spectrum. In either case, host peptidases cleave the peptide to release the C-terminal APPA warhead after uptake, resulting in growth inhibition.¹⁰

Early *in vivo* antagonist experiments with various amino acids suggested that APPA caused growth inhibition by disrupting threonine-related metabolism.^{8, 10} *In vitro* experiments later revealed APPA to be an irreversible inhibitor of the threonine biosynthetic enzyme ThrC, demonstrating saturation kinetics with an apparent k_{inact} of 1.50 min^{-1} and a K_i of $100 \text{ }\mu\text{M}$.^{2, 3} Plausible mechanisms for inhibition included covalent modification of the enzyme or modification of the PLP cofactor, but the experimental data did not provide support for either of these proposed mechanisms.² In these studies, APPA inhibited the threonine synthase from *E.coli* (*Ec*ThrC) irreversibly and at a molar ratio of 1:1 suggesting that inhibition occurs through formation of a covalent adduct between APPA and the enzyme.^{2, 3} This mode of inhibition is observed in other PLP-dependent enzymes such as inhibition of 1-aminocyclopropane-1-carboxylate synthase by *L*-vinylglycine.¹¹ However, tryptic digestion of *Ec*ThrC and peptide analysis did not reveal any modified peptides.² This finding led the authors to propose a mechanism of inhibition that involves formation of a covalent adduct with the PLP cofactor, a mechanism similar to the one of D-amino acid aminotransferase inhibition by the antibiotic D-cycloserine.^{2, 12, 13} Thus, despite almost fourteen years since the identification of APPA as an inhibitor of ThrC, the molecular details regarding the mechanism of inactivation remain unclear.

ThrC is an essential enzyme for growth of bacteria and fungi, and this enzyme is not present in mammals. Given the widespread occurrence of antibiotic resistance and the impending need for the identification of novel antibiotics, ThrC is a worthwhile microbial target and APPA an attractive therapeutic candidate.¹⁴ As such, understanding the mechanism of

inhibition of ThrC by APPA as well as how the producing organisms overcome APPA toxicity will inform on the clinical potential of APPA.

Previous bioinformatic studies on the rhizoctin biosynthetic gene cluster identified the *rhiB* gene, whose product showed homology to threonine synthases (Figure 1c).¹⁵ Curiously, examination of the *B. subtilis* ATCC6633 genome also reveals the presence of a threonine biosynthetic operon (*thrACB*) coding, which is similar in genomic context and sequence to the typical *Bacillus* threonine synthase involved in primary metabolism (*BsThrC*) (Figure 1c).^{5, 15, 16} Sequence alignment between RhiB and *BsThrC* revealed that they share greater than 25% sequence identity further suggesting that RhiB is a threonine synthase (Figure 1c). These observations prompted us to consider that RhiB could function as an APPA-resistant threonine synthase homolog, which could serve as a self-resistance mechanism to circumvent toxicity in the producing organism.

To assess this hypothesis, we reconstituted the activity of both the housekeeping *BsThrC* and the putative resistant RhiB *in vitro*. We demonstrate that RhiB is a PLP-dependent threonine synthase capable of generating threonine from PHSer. Kinetic assays revealed the enzyme to be 7-fold less efficient at generating threonine as compared to the *BsThrC*. However, while *BsThrC* is sensitive to APPA, RhiB was not subject to inhibition by this compound. We also determine the 2.0 Å resolution co-crystal structure of *BsThrC* in complex with APPA demonstrating that inhibition occurs via the formation of a covalent complex with the bound PLP cofactor. Structure-based analysis of the sensitive and resistant threonine synthases provide a molecular basis for the observed APPA resistance in RhiB. This work uncovers the self-resistance mechanism utilized by *B. subtilis* ATCC6633 against APPA, exemplifying one of many ways by which antibiotic producing bacteria can ensure self-resistance during the biosynthesis of a toxic metabolite.

RESULTS AND DISCUSSION

RhiB is a bona fide threonine synthase insensitive to APPA

For biochemical studies, recombinant RhiB was expressed and purified from an *E. coli* heterologous expression system, and its enzymatic activity was tested *in vitro*. Soluble His₆-RhiB required co-expression with the *E. coli* chaperones *GroEL/GroES*. Significantly, throughout the purification process, His₆-RhiB exhibited a yellow color characteristic of either a bound flavin or PLP cofactor. Threonine synthases characterized to date employ the use of PLP as a cofactor to convert *O*-phospho-Lhomoserine into threonine.^{5, 17, 18} UV-Vis spectroscopy of purified His₆-RhiB revealed the presence of an absorption maximum at 420 nm characteristic of PLP-containing enzymes similar to other characterized threonine synthases (Figure S1). We next sought to determine the ability of this enzyme to convert PHSer into threonine. Upon incubation of His₆-RhiB with PHSer a new product appeared as observed by one-dimensional ¹H NMR with a doublet signal at 1.34 ppm and a *J* coupling constant of 6.4 Hz (Figure 2a). This diagnostic signal is characteristic of the H_γ present in threonine suggesting RhiB to be a threonine synthase.

In addition to RhiB, *B. subtilis* ATCC6633 encodes for an endogenous ThrC (*BsThrC*) present within the threonine biosynthetic operon (Figure 1c). Recombinant *BsThrC* was

purified as an N-terminal His₆ tagged construct and the recombinant enzyme exhibited a UV-Vis absorption maximum of 420 nm indicating this protein also co-purified with PLP (Figure S1). As with RhiB, enzyme activity assays with *Bs*ThrC incubated in the presence of PHSer led to the formation of threonine as observed by ¹H NMR (Figure 2b). Threonine formation was not detected in the absence of enzyme (Figure 2c–d).

To gain more insights into the efficiency of the enzymes in catalyzing the formation of threonine, steady state kinetic parameters for both enzymes were determined (Figure 3). Upon formation of threonine catalyzed by threonine synthases, inorganic phosphate is released as a product of the reaction.^{19–21} Phosphate release was thus measured using a previously reported UV-Vis continuous coupled spectrophotometric assay.²² Recombinant *Bs*ThrC exhibited an apparent k_{cat} of 1.74 s^{−1} and an apparent K_M of 329 μM, while His₆-RhiB displayed an apparent k_{cat} of 0.60 s^{−1} and an apparent K_M of 824 μM.

The presence of two bona fide threonine synthases in *B. subtilis* ATCC6633 suggests that RhiB might be an insensitive variant, which could serve as a self-resistance mechanism to avert APPA toxicity. To test this hypothesis, APPA was purified as previously described¹⁵ and the ability of RhiB and *Bs*ThrC to catalyze threonine formation was assessed in the presence of APPA by measuring PHSer consumption via ³¹P NMR. Regardless of the APPA concentration used (either 5x or 20x molar excess), upon incubation of APPA with RhiB, no change in activity was observed compared to enzyme assays performed in the absence of APPA (Figure 4). Interestingly, incubation of *Bs*ThrC with APPA resulted in the formation of a new spectral feature at ~519 nm (Figure S2). Activity assays performed with *Bs*ThrC in the presence of 5x molar excess APPA resulted in decreased enzymatic activity when compared to assays performed in the absence of APPA (Figure 4). These results suggest that APPA is capable of inhibiting *Bs*ThrC but not RhiB, proving that the latter is an APPA-insensitive threonine synthase.

Structural characterization of *Bs*ThrC in complex with APPA reveals mode of inhibition

To establish the molecular basis by which APPA inhibits threonine synthase activity, we determined the 2.0 Å resolution X-ray crystal structures of *Bs*ThrC in its PLP and PLP-APPA bound states (Table 1). As expected, the overall structure of *Bs*ThrC is highly similar to other threonine synthases sharing the canonical fold-type II of PLP-dependent enzymes.^{23, 24} The overall fold of *Bs*ThrC recapitulates the architecture of other enzymes of the tryptophan synthase family.^{23, 24} The structure consists of an N-terminal α/β domain (domain 1; helices α1, α2, α7-α11 and strands β1, β2 and β7-β10), followed by a second α/β domain (domain 2; helices α3- α6 and strands β3- β6) and a C-terminal tail (helices α12 and α13) that extends from one subunit to the other (Figure 5a). The enzyme active site forms at the interface of the two α/β domains of the same subunit while the C-terminal helix α13 interacts with domain 2 of the other subunit (Figure 5a–b).

Solution studies were consistent with a homodimeric assembly for *Bs*ThrC (Figure S1), and a functional homodimer could be generated using crystallographic symmetry operators (Figure 5a). In this homodimer, one subunit is in an open conformation with the PLP bound to the active site Lys59 through an aldimine linkage while the other subunit is in the closed conformation (Figure 5b–d). In the closed conformation, a ligand was found to be bound to

the cofactor even though no substrate was added during the purification or crystal screening process. However, the crystallization condition contained a mixture of amino acids (0.2 M sodium *L*-Glu, 0.2 M DL-Ala, 0.2 M Gly, 0.2 M DL-Lys HCl, 0.2 M DL-Ser). Modeling and refinement efforts support the judicious placement of Ala as the ligand adjacent to the PLP cofactor (Figure S3). Consistent with this conclusion, prior experiments with *E. coli* threonine synthase show Ala as the substrate for half transamination reactions.² In addition, a phosphate group was also modeled in the active site of the *Bs*ThrC-Ala monomer (Figure S3), which was presumably carried over during purification of the enzyme.

As observed in prior structures of threonine synthases, the enzyme undergoes a conformational change upon binding of the substrate (Ala) to control proton transfers and solvent accessibility during the reaction.^{24–26} The superposition of the open and closed conformations reveals that major changes take place in domain 2 upon substrate binding (Figure 5d). Specifically, the loop that connects strand β 4 with helix α 5 (residues Gly108 - Gly113) is disordered in the absence of substrate but becomes structured in the presence of substrate, with residues Phe112 and Gly113 adopting an α -helical conformation that extends helix α 5. This change is accompanied by a significant movement of strands β 3, β 4, β 5 and helix α 6 resulting in a closure of the active site. Finally, when the substrate binds, residues Asn152 and Ser153 are now within hydrogen bonding distance to the phosphate group of the substrate.

Crystals of *Bs*ThrC in complex with the inhibitor APPA showed an orange color, in agreement with the observed shift in the absorption spectrum of the enzyme after binding of the inhibitor (Figure S2a–b and S4).² The complex crystallized as a dimer of functional dimers, and electron density corresponding to the inhibitor was present in all 4 molecules in the asymmetric unit (Figure 6a). Notably, the structure reveals that APPA binds to *Bs*ThrC by forming a covalent complex with the PLP cofactor, and not with any of the residues in the active site (Figure 6a).

The structure of the *Bs*ThrC-APPA complex reveals a closed conformation, and a superposition with *Bs*ThrC bound to Ala shows that the active sites are nearly identical (Figure 6b). The carboxylate group of APPA is engaged by hydrogen bonding interactions with the hydroxyl group of the highly conserved Ser82 and the main chain amines of the highly conserved Thr83 and Thr86 (Figure 5f and S5). The inhibitor is further anchored by interactions between the phosphonate oxygens and the side chains of the highly conserved Thr86, Asn152, Ser153, Arg158 and Asn186 as well as Lys59, which is the lysine that forms an aldimine linkage with PLP when the enzyme is in the resting state (Figure S5). Finally, Phe132 and Ile240 line the enzyme pocket near the β , γ and δ carbons of APPA further supporting the inhibitor (Figure S5). Hence, APPA inhibits threonine synthase by mimicking the physiological substrate, binding to the cofactor and trapping the enzyme in the closed conformation and our studies provide the first direct evidence for this previously proposed mechanism of inhibition.²

Structural comparison of *Bs*ThrC and RhiB active sites reveals the molecular basis of RhiB resistance to APPA

As no active site residues in *Bs*ThrC are involved in covalent interactions with the inhibitor, this raises the question as to why APPA is unable to inhibit RhiB. Presumably, APPA could bind to the PLP co-factor in RhiB as it does to *Bs*ThrC. To address this question, we generated a homology model for RhiB using the *Bs*ThrC-APPA structure as a template and compared their active sites. The only notable difference was the substitution of Phe132 in *Bs*ThrC with Tyr188 in RhiB (Figure 6c). In *Bs*ThrC, Phe132 is ~3.8 Å away from the δ carbon of the phosphonic group of APPA, which is the position of the oxygen of the phosphate group in PHSer (Figure 6d). This led us to the hypothesis that introduction of a hydroxyl group in that position may affect binding of the inhibitor.

To investigate this hypothesis in detail, we generated the Phe132Tyr_*Bs*ThrC and Tyr188Phe_RhiB variants and performed activity assays in the absence and presence of APPA. The substitution of a Phe in Tyr188Phe_RhiB resulted in decreased enzymatic activity in the presence of 5x molar excess of APPA when compared to assays performed in the absence of inhibitor (Figure 4). Similarly, the substitution of a Tyr in Phe132Tyr_*Bs*ThrC resulted in a variant that was less sensitive to the inhibitor compared to the wild-type enzyme (Figure 4). These results suggest that the introduction of a hydroxyl group near the δ carbon of APPA affects its binding to PLP and the substitution of Phe132 in *Bs*ThrC with a Tyr188 in RhiB contributes to resistance to APPA in the latter enzyme.

CONCLUDING REMARKS

We show here that *Bacillus subtilis* ATCC6633 possesses two threonine synthases, one that is sensitive (*Bs*ThrC) and one that is resistant (RhiB) to APPA, the active component of the rhizoctin natural product produced by this organism. In addition, our structural-sequence analyses between RhiB and a 2.0 Å resolution crystal structure of *Bs*ThrC in complex with APPA suggested that a single amino acid substitution in the RhiB active site is responsible for rendering this ThrC homolog resistant to APPA. In support of this finding, we generated a *Bs*ThrC variant tolerant to APPA by introducing a single site mutation within its active site (Phe132Tyr_*Bs*ThrC variant). Together, this work uncovers the self-resistance mechanism utilized by *B. subtilis* ATCC6633 against APPA; the rhizoctin producing organism evades APPA toxicity by employing a second threonine synthase (RhiB) that is insensitive to APPA.

MATERIALS AND METHODS

General Procedures, Chemicals, and Reagents

All chemical reagents were purchased from Sigma-Aldrich unless otherwise noted. All of the materials used for protein production and purification were purchased from GE Healthcare. *Escherichia coli* DH5 α and BL21 (DE3) strains were used for plasmid maintenance and protein overexpression respectively, unless otherwise noted. Activity assays and kinetic characterization of *Bs*ThrC and RhiB were performed with hexahistidine tagged constructs. Nuclear magnetic resonance (NMR) spectra were recorded at 25 °C on an Agilent DD2 600 MHz spectrometer (600 MHz for ^1H and 243 MHz for ^{31}P) equipped with

a OneNMR Probe. Proton and Phosphorous shifts are reported in δ values relative to an external standard of 0.1% tetramethylsilane or 85% phosphoric acid, respectively. Spectra were processed and analyzed using MestReNova 7 software. All NMR analyses were carried out in the Carl R. Woese Institute for Genomic Biology Core Facilities.

Cloning and Site-Specific Mutagenesis

Wild type *BsthrC* (Uniprot ID: A8HUA2, GenBank accession number [EFG91998.1](#) - locus ADGS01000021) and *rhiB* (Uniprot ID: D4HRH9, GenBank accession number [EFG91528.1](#) - locus ADGS01000025) genes were cloned into a pET28b vector carrying an N-terminal His₆ tag following standard cloning protocols. These plasmids were used as templates for the generation of the site-specific mutants by PCR (primers shown in Table S1). The integrity of all recombinant plasmids was confirmed by sequencing (ACGT, Inc.).

Protein Expression and Purification of *BsThrC*

Escherichia coli Rosetta 2(DE3) cells were transformed with expression vectors bearing either wild type or mutant *BsthrC* for heterologous protein production. Briefly, a 2 L Luria-Bertani growth medium supplemented with chloramphenicol (25 μ g/mL) and kanamycin (50 μ g/mL) was inoculated with an overnight 5 mL starter culture. The culture was grown at 37 °C at 230 rpm until the optical density at 600 nm reached 0.6 – 0.8, at which point protein production was induced by addition of 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The culture was then cooled to 18 °C and grown for an additional 18 h. Cells were collected by centrifugation, resuspended in Buffer A (20 mM HEPES-NaOH, pH 8.0, 500 mM NaCl, 10% (v/v) glycerol), and lysed by multiple passages through a C5 Emulsiflex (Avestin) cell homogenizer. Following centrifugation of the lysate, the supernatant was applied to a 5 mL His-Trap (GE Biosciences) column that was previously equilibrated with Buffer B (20 mM HEPES-NaOH, pH 8.0, 1 M NaCl, 30 mM imidazole, 10% (v/v) glycerol). The column was extensively washed with the same buffer and elution of specifically bound protein was carried out using a gradient of increasing imidazole concentration. Fractions containing protein of the highest purity (as determined by SDS-PAGE) were pooled and 5 mM pyridoxal 5'-phosphate (PLP) was added. After 1 h of incubation with PLP, samples were passed through a PD-10 desalting column (GE Healthcare) for buffer exchange (20 mM HEPES-NaOH, pH 8.0, 300 mM NaCl, 10% glycerol). For activity assays, samples were instead purified by size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg (GE Life Sciences) pre-equilibrated with 20 mM HEPES-NaOH, pH 7.5, 300 mM NaCl, and 10% (v/v) glycerol. For crystallization experiments, after the Ni²⁺ affinity column, the polyhistidine affinity tag was removed by overnight incubation with thrombin at 4 °C. Samples were further purified using size exclusion chromatography (Superdex HiLoad 75 16/60) in a buffer of 20 mM HEPES-NaOH, pH 7.5, 300 mM KCl. All samples were concentrated using 10 kDa molecular weight cutoff (MWCO) Amicon centrifugal filters, flash frozen in liquid nitrogen and stored at –80 °C. Protein concentrations were determined spectrophotometrically by measuring the absorbance at 280 nm, and using the molecular weights and extinction coefficients calculated by the ExPASy ProtParam tool (Table S2).²⁷

Protein Expression and Purification of RhiB

E. coli BL21 (DE3) cells were co-transformed with *pGro7* (chaperone co-expression plasmid) and expression vectors bearing either wild type or mutant *rhiB* for heterologous protein production. Briefly, a 2L Terrific Broth growth medium (12 g tryptone, 24 g yeast extract, 4 mL glycerol and 100 mL of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 per Liter) supplemented with chloramphenicol (25 $\mu\text{g}/\text{mL}$) and kanamycin (50 $\mu\text{g}/\text{mL}$) was inoculated with an overnight 5 mL starter culture. The culture was grown at 37 °C and 230 rpm until the optical density at 600 nm reached 0.8 – 1.0, at which point protein production was induced by addition of 0.2–0.3 mM IPTG and 0.08–0.4% (w/v) L-arabinose. The culture was then cooled to 18 °C and grown for an additional 18 h. Cells were collected by centrifugation, resuspended in Buffer A and lysed by multiple passages through a C5 Emulsiflex (Avestin) cell homogenizer. Following centrifugation of the lysate, the supernatant was applied to a 5 mL His-Trap (GE Biosciences) column that was previously equilibrated with Buffer B. Fractions containing protein of the highest purity (as determined by SDS-PAGE) were pooled and 5 mM pyridoxal 5'-phosphate (PLP) was added. After 1 h of incubation with PLP, samples were passed through a PD-10 desalting column (GE Healthcare) for buffer exchange (20 mM HEPES-NaOH, pH 8.0, 300 mM NaCl, 10% (v/v) glycerol). For activity assays, samples were instead purified by size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg (GE Life Sciences) pre-equilibrated with 20 mM HEPES-NaOH, pH 7.5, 300 mM NaCl, and 10% (v/v) glycerol. All samples were concentrated using 10 kDa MWCO Amicon centrifugal filters, flash frozen in liquid nitrogen and stored at –80 °C. Protein concentrations were determined by UV-Visible spectroscopy using molecular weights and extinction coefficients at 280 nm calculated by the ExPASy ProtParam tool (Table S2).²⁷

Analytical Size Exclusion Chromatography of BsThrC and RhiB

The oligomerization state of *BsThrC* and *RhiB* was determined by analytical size exclusion chromatography using a Phenomenex 3000 Yarra column (300 × 4.6 mm) pre-equilibrated with 20 mM HEPES-NaOH, pH 7.5, 300 mM KCl at 0.5 mL min⁻¹ coupled to an Agilent HPLC 1200 Infinity Series LC instrument. Protein elution was monitored by measuring the absorbance at 280 nm. A calibration standard curve was generated to determine proper oligomerization sizes according to the manufacturers protocol (GE Life Sciences).

BsThrC and RhiB Activity Assays

The following conditions were used to determine the ability of *BsThrC* and *RhiB* to catalyze threonine formation: 20 mM HEPES-NaOH pH 7.5, 1 mM PHSer, 50 μM enzyme (either *BsThrC* or *RhiB*), and 5 μM PLP in a final volume of 500 μL and samples were incubated for 2 h at 37 °C. Following the incubation period, proteins were removed using 3 kDa MWCO Amicon centrifugal filters (14,000 × g, 4 °C), the filtrate was collected and lyophilized to dryness. Dried solids were then dissolved in 500 μL of D_2O and analyzed via ¹H NMR.

Kinetic Characterization of *BsThrC* and *RhiB*

Michaelis-Menten kinetics were performed utilizing a coupled assay for the continuous monitoring of inorganic phosphate (P_i) release by the enzyme.²² Briefly, in the presence of inorganic phosphate, 2-amino-6-mercapto-7-methylpurine riboside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. This conversion of MESG results in a shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product ($\epsilon_{360} = 11,000 \text{ M}^{-1} \text{ cm}^{-1}$). This shift allows for continuous monitoring of P_i production by measuring the increase in absorbance at 360 nm. The following conditions were used: 50 mM HEPES-NaOH, pH 7.5, 100 μM PLP, 200 μM MESG, 0.00375 U/ μL PNP, either 0.25 μM *RhiB* or 0.1 μM *BsThrC*, and various concentrations of *O*-phospho-L-homoserine (PHSer). Measurements were performed in triplicates.

Purification of rhizoctin and APPA from *B. subtilis* 6633

Purification of rhizoctin was performed as previously described.¹⁵ Following purification, acid hydrolysis with 6 M HCl was performed to obtain APPA following previously published procedures.^{6, 10}

Binding of APPA

Binding of the inhibitor to the enzyme was monitored spectrophotometrically by monitoring the formation of a chromophore at $\sim 500 \text{ nm}$.² *BsThrC* and *RhiB* were incubated with APPA (488 μM) in a 2:1 ratio for 15 min. Following the incubation period, both proteins were washed 5 times using 10 kDa MWCO Amicon centrifugal filters (500 μL , $14,000 \times g$, 4 $^{\circ}\text{C}$, 20 min) with buffer (20 mM HEPES-NaOH, pH 7.5, 300 mM NaCl and 10% (v/v) glycerol), and the absorption spectra were recorded on a Cary 4000 UV-Visible spectrophotometer at ambient temperature.

Crystallization of *BsThrC*

Initial crystallization conditions were determined by the sparse matrix sampling method using commercial screens. Crystals of *BsThrC*-APPA were grown using the hanging drop vapor diffusion method. Briefly, 1 μL of protein at 4 mg/mL concentration was incubated with 15 molar equivalents of APPA (1.5 mM) for 1.5 h at ambient temperature, mixed with 1 μL of precipitant solution (0.03 M MgCl_2 hexahydrate, 0.03 M CaCl_2 dihydrate, 18% v/v PEG 550 MME, 9% w/v PEG 20,000, 0.1 M MES/imidazole, pH 6.5), and equilibrated over a well containing the same precipitant solution at 9 $^{\circ}\text{C}$. The crystals were sequentially soaked in precipitant solution supplemented with increasing concentrations of 20 and 30% ethylene glycol prior to vitrification by direct immersion in liquid nitrogen.

The *BsThrC*-Ala/*BsThrC*-PLP crystals were pooled from the initial screening trays. Briefly, 0.2 μL of protein at 6 mg/mL was mixed with 0.2 μL of MORPHEUS crystallization screen²⁸ condition H1 (0.2 M sodium L-glutamate, 0.2 M DL-alanine, 0.2 M glycine, 0.2 M DL-lysine HCl, 0.2 M DL-serine, 10% w/v PEG 20 000, 20% v/v PEG MME 550, 0.1 M MES/imidazole, pH 6.5) using the sitting drop vapor diffusion method, and equilibrated over a well containing the same precipitant solution at 9 $^{\circ}\text{C}$. The crystals were sequentially soaked

in precipitant solution supplemented with increasing concentrations of 20 and 30% ethylene glycol prior to vitrification by direct immersion in liquid nitrogen.

Data Collection, Phasing and Structure Determination

X-ray diffraction data were collected at Life Sciences Collaborative Access Team (LSCAT), Sector 21, Argonne National Laboratory. All data were indexed, integrated and scaled using AutoProc.²⁹ Both structures were determined by molecular replacement as implemented in the Phenix program suite;³⁰ for the *Bs*ThrC-PLP/*Bs*ThrC-Ala structure, the coordinates of threonine synthase from *Aquifex aeolicus* Vf5 (59% sequence identity, 77% sequence similarity, 97% cover, PDB ID: 2ZSJ) were used as a search model, while for the *Bs*ThrC-APPA complex the refined coordinates of *Bs*ThrC-PLP/*Bs*ThrC-Ala were used. In both cases, the resultant solutions were subsequently used as starting models for several rounds of automated model building using Phenix Autobuild³¹ and Buccaneer^{32–34}, followed by rounds of manual rebuilding using Coot³⁵, and refinement using either Phenix Refine³⁶ or REFMAC5³⁷. Ligands were built in Coot³⁵ and the resultant models were further refined and manually inspected. In the final stages of refinement, water molecules were added with Phenix Refine and confirmed by manual inspection. In all cases, the quality of the in-progress model was routinely monitored using both the free R factor³⁸ and MolProbity³⁹ for quality assurance.

Homology Modeling of RhiB

The structure of the RhiB protein was modeled from its primary amino acid sequence by using the structure-based homology modeling tools Phyre2⁴⁰ and SWISS-MODEL.^{41, 42} For the Phyre2 model, the intensive mode of Phyre2 was selected and a model was generated based on several templates. For the SWISS-MODEL generated models, several different templates were used (PDB IDs: 2C2G, 3AEY, 3AEX) including the *Bs*ThrC-APPA and *Bs*ThrC-PLP since a conformational change that closes the active site occurs upon binding of the substrate. All models were superimposed with each other and with the *Bs*ThrC-APPA structure and were used to identify differences between the active sites of *Bs*ThrC and RhiB. The active sites of all models were identical. Differences were observed between the models generated by PLP-bound templates and the models generated by templates in a closed conformation. The model generated by SWISS-MODEL using the *Bs*ThrC-APPA structure as template was finally selected and is shown in Figure 6c–d.

Inhibition Assays – ³¹P NMR

Enzyme inhibition assays were carried out at 30 °C in 50 mM HEPES-NaOH (pH 7.5). In a final volume of 400 µL, 0.5 µM holo-*Bs*ThrC wild type and mutant was pre-incubated with 2.5 µM APPA for 1 h at 30 °C, and then 1.6 mM PHSer (~5 x K_M) was added. For RhiB wild type and mutant, 3 µM holoenzyme was pre-incubated with 15 µM or 60 µM APPA for 1 h at 30 °C, and then 4 mM PHSer (~5 x K_M) was added. After 30 min incubation of the enzymes with PHSer, the samples were passed through 10 kDa MWCO Amicon centrifugal filters, and 300 µL of the filtrate were removed and mixed with 300 µL D₂O. For quantification during ³¹P NMR, 0.5 mM of dimethylphosphinic acid was added as internal standard in all samples, and PHSer consumption was measured for the calculation of enzyme activity. The activity of the enzymes in the presence of APPA was expressed as

“percentage of PHSer consumed” in respect to the corresponding wild type reaction that contained no APPA (considered as 100%). All measurements were performed in triplicates.

Estimation of PLP Content – Holoenzyme Concentration

Protein concentrations were determined by measuring the absorbance at 280 nm using the molecular weights and extinction coefficients calculated by the ProtParam tool (ExPASy server) (Table S3).²⁷ In order to calculate the concentration of holo-RhiB and holo-*Bs*ThrC in the protein preparations, the absorbance at 388 nm before (free PLP) and after (total PLP) the addition of 0.2 M NaOH was measured, as previously described.^{43–46} The difference between the two values corresponds to the PLP concentration that is bound to the enzyme (holoenzyme concentration). Free PLP has a maximum absorbance at 388 nm with an extinction coefficient of 6,600 M⁻¹ cm⁻¹ in 0.1 M NaOH.⁴⁶ The ϵ_{388} of free PLP in 0.2 M NaOH was measured by using 40 μ M and 80 μ M PLP and was found to be $\sim 6,525$ M⁻¹ cm⁻¹. All measurements were performed in triplicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the staff at Life Sciences Collaborative Access Team (LS-CAT, Argonne National Laboratory, Argonne, IL) for facilitating data collection. We also thank Kou-San Ju, Michelle N. Goettge, Elizabeth Parkinson, Madeline M. López-Muñoz, and Kwo-Kwang for helpful discussions. Funded by NIH grants PO1GM077596 (to S.K.N. and W.W.M.) and R01GM127659 (to WWM).

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Figure 1.

Overview of rhizocticin biosynthetic gene cluster. (a) Reaction catalyzed by threonine synthases (*Bs*ThrC), which is inhibited by APPA. (b) Structure of the phosphono-oligopeptides rhizocticin and plumbemycin. The non-proteinogenic phosphono-amino acid (*Z*)- *L*-2-amino-5-phosphono-3-pentenoic acid is shown in red. (c) Rhizocticin biosynthetic gene cluster. Biosynthetic genes are shown in red and the putative gene involved in self-resistance, RhiB, is shown in green. For comparison, the *B. subtilis* ATCC 6633 threonine biosynthetic operon is shown below indicating the percent amino acid sequence identity between the predicted threonine synthase homolog RhiB and the *Bs*ThrC.

Figure 2.

In vitro reconstitution of His₆-RhiB activity. ¹H NMR spectra of (a, b) reaction assays following incubation of PHSer with either (a) His₆-RhiB or (b) His₆-BsThrC, a (c) threonine standard solution, and a (d) reaction assay with no enzyme present. Only the upfield portion of the ¹H NMR spectrum is shown for clarity.

Figure 3.

Kinetic characterization of His₆-RhiB and His₆-BsThrC. (a) His₆-BsThrC and (b) His₆-RhiB activity was measured by determining P_i release from *O*-phosphohomoserine. Enzymatic rates were plotted as a function of *O*-phosphohomoserine concentration and the data were fit to the Michaelis–Menten equation. Results are means ± standard error of the mean (SEM) of triplicate experiments.

Figure 4.

BsThrC and *RhiB* activity in the presence of APPA. End point assays were performed by measuring the amount of PHSer consumed at the end of the reaction. Data are plotted as the means $\pm$ standard error of the mean (SEM) of triplicate experiments.

Figure 5.

Crystal structure of *BsThrC*. (a) Ribbon diagram of the functional homodimer of *BsThrC* as generated by the space group symmetry operations. In blue is domain 1, in yellow is domain 2 and the C-terminal tail is shown in red. The helix $\alpha 13$ of one monomer interacts with the domain 2 of the other monomer. (b) Surface representation of the functional dimer. In blue is the *BsThrC*-PLP subunit in the open conformation, and in pink is the *BsThrC*-Ala subunit in the closed conformation. (c, d) Zoomed in view of the surface in the open and closed conformations, respectively. Upon binding of the substrate, domain 2 (shown in gray) moves and closes the active site. Grey: domain 2, blue: domain 1 in the open state, pink: domain 1 in the closed state. (e) Conformational changes of domain 2 upon binding of the substrate. Only domain 2 is shown. Blue: open conformation, pink: closed conformation, dashed line: residues missing from crystal structure. (f, g) Stick representation of residues that interact with the bound ligands (alanine and phosphate, respectively) showing their position before (blue) and after (pink) binding. Dashed lines represent hydrophobic interactions and solid lines represent hydrogen bonding interactions. Residues that interact with the cofactor are not shown.

Figure 6.

Crystal structure of *BsThrC*-APPA. (a) Difference Fourier map (F_o-F_c) contoured to 2.0σ (blue) showing the APPA bound to the cofactor (adduct colored in orange). It is evident from the electron density that the APPA is not covalently bound to any active site residues. (b) Superposition of the *BsThrC*-APPA (teal) and *BsThrC*-Ala (pink) active sites. The two active sites are identical further supporting the hypothesis that APPA inhibits threonine synthase by “trapping” the enzyme in the closed conformation. The PLP-APPA adduct is shown in orange and the PLP-Ala adduct in yellow. (c) Superposition of *BsThrC*-APPA (teal) and *RhiB* (yellow) active sites. The *RhiB* model was generated by SWISS-MODEL⁴² using the *BsThrC*-APPA structure as a template. The labels correspond to the *RhiB* residues. (d) Phe132 in *BsThrC* is $\sim 3.8 \text{ \AA}$ away from the C δ of APPA. In *RhiB*, the corresponding Phe has been substituted by Tyr. (e) Sequence alignment of *BsThrC* and *RhiB*. Star: lysine that forms the internal aldimine, circles: residues that interact with APPA, squares: residues that interact with the cofactor.

Table 1.

Data collection and refinement statistics.

	<i>Bs</i> ThrC-PLP/ <i>Bs</i> ThrC-Ala (6CGQ)	<i>Bs</i> ThrC-APPA (6NMX)
Data collection		
Space Group	C222 ₁	P2 ₁
a, b, c (Å), β (°)	49.8, 127.9, 237.1	49.5, 104.2, 125.6, 99.6
Resolution (Å)	118.5 – 2.0	123.8 – 1.97
R _{sym} (%) ^[a,b]	12.7 (102.7)	12.9 (85.2)
I/σ(I)	14.6 (2.2)	10.7 (2.1)
Completeness (%)	100 (97.6)	91.3 (95.7)
Redundancy	8.2 (8.4)	4.7 (4.7)
Total reflections	411,421	379,109
Unique reflections	50,330	80,926
Refinement		
Resolution (Å)	49.7–2.0	123.8 – 1.97
No. reflections used	50,265	80,926
R _{work} / R _{free} [%]	0.18/0.23	0.16/0.21
Number of atoms		
Protein	5,121	10,437
PO4	5	
PLP	15	
PLP-Ala	21	
PLP-APPA		108
Water	370	849
B-factors		
Protein	28.23	22.93
PO4	25.91	22.44
PLP	32.52	
PLP-Ala	21.08	
PLP-APPA		18.61
Water	34.41	29.55
R.m.s deviations		
Bond lengths (Å)	0.007	0.007
Bond angles (°)	0.873	0.916
MOLPROBITY statistics		
Clash score	2.71	4.13
Ramachandran outliers/ allowed/ favored (%)	0.29/1.18/98.53	0.29/1.08/98.63

^aHighest-resolution shell is shown in parenthesis^bR_{sym} = Σ|I_i - <I>| / ΣI_i, where I_i = intensity of the ith reflection and <I> = average intensity of symmetry-related observations of a unique reflection

^cR-factor = $\Sigma |F_{\text{Obs}} - F_{\text{Calc}}| / \Sigma |F_{\text{Obs}}|$ and R-free is the R value for a test set of reflections consisting of a random 5% of the diffraction data not used in refinement.

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