A Unique Hydrophobic Cluster Near the Active Site Contributes to Differences in Borrelidin Inhibition among Threonyl-tRNA Synthetases*

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Borrelidin, a compound with anti-microbial and anti-angiogenic properties, is a known inhibitor of bacterial and eukaryal threonyl-tRNA synthetase (ThrRS). The inhibition mechanism of borrelidin is not well understood. Archaea contain archaeal and bacterial genre ThrRS enzymes that can be distinguished by their sequence. We explored species-specific inhibitor inhibition of ThrRSs. The activity of ThrRS from Sulfolobus solfataricus and Halobacterium sp. NRC-1 was inhibited by borrelidin, whereas ThrRS enzymes from Methanococcus jannaschii and Archaeoglobus fulgidus were not. In Escherichia coli ThrRS, borrelidin binding induced a conformational change, and threonine was not activated as shown by ATP-PP, exchange and a transient kinetic assay measuring intrinsic tryptophan fluorescence changes. These assays further showed that borrelidin is a noncompetitive tight binding inhibitor of E. coli ThrRS with respect to threonine and ATP. Genetic selection of borrelidin-resistant mutants showed that borrelidin binds to a hydrophobic region (Thr-307, His-309, Cys-334, Pro-335, Leu-489, Leu-493) proximal to the zinc ion at the active site of the E. coli ThrRS. Mutating residue Leu-489 → Trp reduced the space of the hydrophobic cluster and resulted in a 1500-fold increase of the $K_i$ value from 4 nM to 6 μM. An alignment of ThrRS sequences showed that this cluster is conserved in most organisms except for some Archaea (e.g. M. jannaschii, A. fulgidus) and some pathogens (e.g. Helicobacter pylori). This study illustrates how one class of natural product inhibitors affects aminoacyl-tRNA synthetase function, providing potentially useful information for structure-based inhibitor design.

Aminoacyl-tRNA synthetases (aaRSs) catalyze the acylation of transfer RNAs with their cognate amino acids and are therefore essential enzymes for protein synthesis in all organisms (1). They display exquisite specificity in discriminating between similar amino acid or tRNA substrates. Even though the core architectures of the active sites of individual aaRSs are relatively conserved, protein sequence alignments showed significant divergence among the three domains of life. However, finely tuned structural differences between aaRS orthologs provide ample opportunities for the evolution of species-specific inhibitors of any given tRNA synthetase. For example, mupirocin, an antibiotic produced by Pseudomonas fluorescens, selectively inhibits the prokaryotic isoleucyl-tRNA synthetases but has little or no effect on the eukaryotic enzymes (2, 3). Indolmycin, a secondary metabolite of Streptomyces griseus and analogue of tryptophan, inhibits only one of the two tryptophanyl-tRNA synthetases in Streptomyces coelicolor (4).

ThrRS is a class II aaRS containing an N-terminal editing domain, a C-terminal tRNA binding domain, and a zinc-binding catalytic domain with the class II conserved motifs 1, 2, and 3 that provide critical ATP binding determinants (5). ThrRSs from Chinese hamster ovary cells, Saccharomyces cerevisiae and Escherichia coli (reviewed in Ref. 6) are specifically inhibited by borrelidin, an 18-membered macrolide-polyketide (Fig. 1) produced by Streptomyces spp (7–9). Borrelidin was shown to have anti-malarial activity (10). Furthermore, borrelidin was found to interfere with capillary tube formation, possibly through anti-angiogenesis effects that are mediated through the ThrRS inhibition and the caspase activation pathways (11). Borrelidin also inhibited a S. cerevisiae cyclin-dependent kinase (Cdc28/Cln2), an indication of anti-tumor activity (12). Gene expression profiling of S. cerevisiae revealed that borrelidin up-regulated GCN4 leucine zipper mRNA synthesis (13), and this in turn induced the expression of amino acid biosynthetic enzymes. Because of its intriguing biological activities, the chemical synthesis (14, 15) and biosynthesis (16) of borrelidin have been explored.

Presumably as a consequence of borrelidin inhibition of human ThrRS, the compound may also have undesired cytotoxic effects. To develop borrelidin into an important anti-microbial, anti-angiogenesis, and anti-tumor drug candidate, a fuller understanding of the mechanism of borrelidin inhibition of ThrRS is required. Previous reports showed that borrelidin is a non-competitive inhibitor with respect to threonine (17) and inhibi-

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A. fulgidus) e.g. M. jannaschii, organisms except for some Archaea (sequences showed that this cluster is conserved in most E. coli. 

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1 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; ThrRS, threonyl-tRNA synthetase.
Borrelidin Binding Site of ThrRS Enzymes

its the transfer of activated threonine to tRNA\(^{Thr}\) (18). Characterization of borrelidin resistant mutants showed that resistance resulted from ThrRS overexpression in vivo (19) or from a mutation affecting the \(K_m\) of threonine (17). However, the binding site is not known. Here we report the difference in borrelidin inhibition of the recombinant ThrRSs from Methanothermococcus jannaschii, Sulfolobus solfataricus, Halobacterium spp., and Archaeoglobus fulgidus as well as the identification of the binding site of ThrRS.

EXPERIMENTAL PROCEDURES

General—[\(^{1}[\text{H}]\)threonine, \([\text{U}^{13}C\]threonine, and \([\text{U}^{15}S]^{32}P\]pyrophosphate were from Amersham Bioscience. Escherichia coli total RNA was from Sigma. The TOPO-TA cloning kit was from Invitrogen. The QuikChange system. A library of random clones was cloned into the expression vectors described below. Specific mutant vector. After verification of the DNA sequences, the genes were subcloned into the pCBS vector between the KpnI and EcoRI sites.

Cloning and Site-directed Mutagenesis of thrS Genes—Primers were designed to PCR-amplify each open reading frame and introduce desired restriction sites. The forward primers contained a restriction site and 20 nucleotide identical to the start sequence of the 5′-end; the designed to PCR-amplify each open reading frame and introduce downstream sequences.

Fig. 1. Chemical structure of borrelidin.

Experimental Design—The TOPO-TA cloning kit was from Invitrogen. The QuikChange system. A library of random clones was cloned into the expression vectors described below. Specific mutant alleles were generated by site-directed mutagenesis using the QuikChange system. The library of mutant genes was generated by error-prone PCR using the Mutatase kit. For in vivo tests, the genes were subcloned into the pCBS vector between the KpnI and BglIII sites under trpS promoter control. For protein expression, the genes were cloned into pET20b vectors using the XbaI and BamHI sites.

In Vivo Testing for ThrRS Activity and Borrelidin Inhibition—ThrRS activity was tested by complementation of E. coli thrS\(^S\) strain with thrS mutants at 42 °C on minimal medium containing casamino acids. Borrelidin-resistant genes were selected on minimal medium plate containing borrelidin. The E. coli thrS mutant library was transformed into W3110, and the transformants were grown in glucose M56 minimal medium to the sequence of the 3′-end. PCR-amplified thrS open reading frames from M. jannaschii, S. solfataricus, Halobacterium sp. NRC-1, A. fulgidus, and E. coli DNAs were cloned into the pCR2.1-TOPO vector. After verification of the DNA sequences, the genes were subcloned into the expression vectors described below. Specific mutant alleles were generated by site-directed mutagenesis using the QuikChange system. The library of mutant genes was generated by error-prone PCR using the Mutatase kit. For in vivo tests, the genes were subcloned into the pCBS vector between the KpnI and BglIII sites under trpS promoter control. For protein expression, the genes were cloned into pET20b vectors using the XbaI and BamHI sites.

Aminoacylation of tRNA—Thr-tRNA formation was measured by acid-precipitable radioactivity (21) in 0.1 ml of reaction mixtures containing 60 nm Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KC\(_2\), 5 mM dithiothreitol, 0.01–10 mM [\(^{13}C\)threonine (200 pmol), and 1 unit inorganic pyrophosphatase in a total volume of 0.1 ml at 37 °C for 15–20 min. The wild-type ThrRS enzymes were 80–100% active, whereas the activity of the mutant enzymes varied from 0.1–100% relative activity. These values were used to determine enzyme concentrations for calculation of \(K_m\). The enzymes were stored in 40% glycerol buffer (60 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KC\(_2\), 5 mM dithiothreitol) at –20 °C.

Aminoacylation of tRNA—Thr-tRNA formation was measured by acid-precipitable radioactivity (21) in 0.1 ml of reaction mixtures containing 60 nm Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KC\(_2\), 5 mM dithiothreitol, 0.01–10 mM ATP, 0.01–1 mM [\(^{32}P\)threonine, 4 mg/ml total tRNA (E. coli tRNA for E. coli ThrRSs and M. jannaschii tRNA for other ThrRSs), 0–6000 nm borrelidin, and 0.1–100 nm enzyme. Reactions were carried out in triplicate at 37 °C for E. coli and Halobacte- rium ThrRS, at 55 °C for S. solfataricus ThrRS, and 65 °C for M. jannaschii and A. fulgidus ThrRS. For steady state kinetic constants \(K_m\) and \(k_{cat}\) measurement, the enzymes were diluted to obtain linear initial velocities. \(K_m\) and \(k_{cat}\) were calculated in KaleidaGraph 3.0 using a Michaelis-Menten equation fit. For \(K_m\), measurements, saturating amounts of substrates (0.5 mM threonine, 1 mM ATP, 4 mg/ml tRNA) were mixed with various amounts of borrelidin (0–6 μM). The reaction mixture (ATP, tRNA, ThrRS enzyme) was preincubated in the presence of borrelidin at 37 °C for 3 min, and then threonine was added to initiate the reaction. All reactions were done in triplicate and repeated at least twice. The initial velocities obtained were plotted against borrelidin concentration to obtain \(IC_{50}\) values. The apparent \(K_{app}\) values were calculated based on the simplified equation (\(K_{app} = IC_{50}/E/2\)) derived (22) for noncompetitive tight binding inhibition.

ATP-PP\(_1\), Exchange Assay—Threonine activation was measured by quantitation of \([\gamma-^{32}P]\)ATP retained on activated carbon (13) in 0.2 ml of reaction mixtures containing 60 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 30 mM KC\(_2\), 5 mM dithiothreitol, 2 mM ATP, 0.01–1 mM threonine, 1 mM KF, 2 mM [\(^{32}P\)]pyrophosphate (4 cpm/μmol), 0–2400 nm borrelidin, and 1–100 nm enzyme. The kinetic constants were obtained as described above for the amnoacylation reactions.

Presteady State Analysis—Intrinsically tryptophan fluorescence changes of the E. coli ThrRS enzyme upon ligand binding were measured by monitoring the excitation and emission spectra (23). The reactions contained 20 mM HEPES, pH 7.4, 150 mM KC\(_2\), 15 mM MgCl\(_2\), 1 μM ThrRS enzymes, and various substrates. The binding constants were measured by rapidly mixing ThrRS enzyme with various amounts of borrelidin (0–8 μM) at 30 °C. Inhibition of adenylation was measured by rapidly mixing ThrRS enzyme with threonine (0.5 mM), ATP (1.5 mM), and various amount of borrelidin (0–8 μM) at 30 °C.

RESULTS AND DISCUSSION

Borrelidin Inhibition of Archaeal and Bacterial ThrRS Enzymes—Phylogenetic analysis of ThrRS sequences (24, 25) showed the existence of markedly different bacterial/eukaryal and archaeal versions of ThrRS. There are large deletions or additions in the N-terminal region of these enzymes; archaeal ThrRS has a shorter N-terminal region, an insertion domain at motif 3 (26), and many conserved differences in the catalytic domain. In this work we investigated the S. solfataricus and Halobacterium sp. NRC-1 ThrRS, which are more closely related to the bacterial enzyme, and the archaeal enzyme from A. fulgidus and M. jannaschii. The influence of borrelidin on the enzyme activity was measured in the amnoacylation assay. As shown in Fig. 2, ThrRS from M. jannaschii and A. fulgidus were not inhibited at 1 μM borrelidin, whereas those of S. solfataricus and Halobacterium were inhibited. To compare these enzymes under similar conditions, the ATP-PP\(_1\) exchange reaction was used for steady-state kinetic measurements. As shown in Table I, the tested ThrRSs had similar \(K_m\) values for threonine, but the \(k_{cat}\) values of ThrRS from M. jannaschii and A. fulgidus were lower than those of the S. solfataricus and E. coli enzymes. The inhibition constants \(K_{i}\) for M. jannaschii and A. fulgidus ThrRSs were at least 1500 times higher than that of S. solfataricus ThrRSs, indicating that the M. jannaschii and A. fulgidus enzymes may not bind borrelidin.

The N-terminal Editing Domain Does Not Contribute to Borrelidin Binding—No sequence homology of the N-terminal do-
main was observed between the borrelidin-inhibited ThrRS and the uninhibited type. Also, _S. solfataricus_ contains two thrS related genes (25, 27); the archaeal genre ThrRS lacks the putative catalytic domain, and the bacterial genre ThrRS lacks most of the entire N-terminal editing domain. The bacterial genre _S. solfataricus_ ThrRS can be inhibited by borrelidin. To determine whether the N-terminal region contributes to borrelidin binding, we aminoacylated _E. coli_ tRNA with wild-type _E. coli_ ThrRS and _M. jannaschii_ tRNA with the _A. fulgidus_ enzyme in the presence or absence of borrelidin. A similar experiment was carried out with the corresponding N-terminal domain-deleted enzymes. As shown in Fig. 3, the _E. coli_ N-terminal-deleted ThrRS was inhibited by borrelidin to the same extent as the wild-type enzyme; the _A. fulgidus_ N-terminal-deleted ThrRS remained uninhibited. Therefore, the N-terminal domain does not contain a borrelidin binding site.

**Table I**

<table>
<thead>
<tr>
<th>Source of ThrRS</th>
<th><em>K_m (Thr)</em></th>
<th><em>k_cat</em> (s⁻¹)</th>
<th><em>K</em>{app} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>10 ± 5</td>
<td>33 ± 3</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td><em>S. solfataricus</em></td>
<td>100 ± 5</td>
<td>16 ± 1</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td><em>A. fulgidus</em></td>
<td>90 ± 5</td>
<td>3.8 ± 0.3</td>
<td>&gt;6000</td>
</tr>
<tr>
<td><em>M. jannaschii</em></td>
<td>100 ± 5</td>
<td>1.2 ± 0.1</td>
<td>&gt;6000</td>
</tr>
</tbody>
</table>

To compare the enzymes under the same experiment conditions, kinetic data were determined by ATP-PPi exchange reactions. To compare the kinetic data with the literature value, the apparent rate constants were plotted with respect to borrelidin concentration. The apparent first order rate constants from Fig. 4A versus borrelidin concentration. Error bars indicate the 95% confidence limit. The slope and y-intercept represent estimates of _k_on_ and _k_off_ respectively.
inhibit the second step, the transfer of activated threonine to tRNA\(^{\text{Thr}}\) (18). However, because borrelidin is a tight binding inhibitor, the IC\(_{50}\) values could vary remarkably depending on the enzyme concentration. Therefore, we measured the IC\(_{50}\) value of the same amount of \(E.\ coli\) ThrRS enzyme determined by both ATP-PP\(_i\) exchange and aminoacylation. ATP-PP\(_i\) exchange reaction measures the kinetic parameter of the first step, threonine activation; aminoacylation determines the overall formation of Thr-tRNATh form. Fig. 5, A and B show that the IC\(_{50}\) values were similar in both reactions; the calculated \(K_i\) for \(E.\ coli\) ThrRS in the ATP-PP\(_i\) exchange reaction (4.2 nM) is very similar to the \(K_i\) of 3.7 nM in aminoacylation, indicating that borrelidin inhibits \(E.\ coli\) ThrRS at the first step of threonine activation and thus the overall aminoacylation. To confirm this, presteady-state kinetic analysis of the ThrRS-catalyzed adenylation reaction was carried out in the presence of borrelidin. Fig. 5C shows transient changes in the intrinsic tryptophan fluorescence of ThrRS in the presence or absence of substrates and inhibitor. All data traces were well described by fits to a single exponential function. In the presence of threonine and ATP, the enzyme fluorescence is quenched by 10%, with half the quench amplitude occurring within the dead time of the instrument (bottom fitted trace). This instantaneous quench reflects the enzyme response to the rapid binding of the substrates, whereas the fitted transient corresponds to an isomerization that precedes the chemical step of adenylation (23). In the absence of substrates the fluorescence change is negligible (top trace). The addition of various concentrations of borrelidin to ThrRS prior to rapid mixing with threonine and ATP abolishes the adenylation-associated quench but not that associated with substrate binding, as expected for a noncompetitive inhibitor. At the highest borrelidin concentration, the fluorescence quench includes a small contribution from the prebound borrelidin in addition to the rapid binding of threonine and ATP. Taken together, both steady-state and presteady-state kinetic data showed that borrelidin inhibits ThrRS at the activation step rather than at the transfer step (18).

**Borrelidin Is a Noncompetitive Inhibitor of \(E.\ coli\) ThrRS with Respect to Threonine and ATP**—All the data above indicated that borrelidin may bind near or at the active site. To examine whether borrelidin has overlapping binding sites with the other enzyme substrates, we evaluated the inhibition mechanism with respect to threonine and ATP. The Michaelis-Menten derived double-reciprocal plot is commonly used to distinguish the inhibition pattern of reversible inhibitors with the assumption that the free inhibitor concentration could approximate the total concentration of the added inhibitor. However, borrelidin is a tight binding inhibitor that inhibits ThrRS at a very low concentration that is close to the enzyme concentration. Under these conditions the concentration of the enzyme-inhibitor complex is not negligible compared with the inhibitor concentration, so we can not use the double-reciprocal plot method to determine the inhibition patterns of borrelidin.

The inhibition pattern was then determined according to the equations derived for tight binding inhibitors (22, 28, 29). Several graphic approaches are available, and the most direct one is to determine the IC\(_{50}\) values for the inhibitor at a fixed enzyme concentration but at different substrate concentrations. For the competitive type, IC\(_{50}\) values increase linearly with increasing substrate concentration; that of the uncompetitive type is linear with the reciprocal value of the substrate concentration, i.e. IC\(_{50}\) values decrease sharply with the increasing substrate concentration. For the noncompetitive type, the relationship between IC\(_{50}\) values and substrate concentration varies depending on the dissociation constant of the inhibitor-enzyme-substrate complex (\(aK_i\)) and the inhibitor-enzyme complex (\(K_i\)). As shown in Fig. 6A, when \(K_i\) equals \(aK_i\), the equation for a tight binding competitive inhibitor reduces to IC\(_{50}\) = \(K_i + [E]/2\), indicating that substrate binding does not influence the binding of inhibitor to ThrRS enzyme. If \(aK_i < K_i\), the inhibitor-enzyme-substrate complex formation is favored over the inhibitor-enzyme complex, indicating that the substrate has a positive influence on inhibitor binding to the enzyme. If \(aK_i > K_i\), the substrate must have a negative influence on inhibitor binding to the enzyme. Each IC\(_{50}\) value shown in Fig. 6, B and C was the average of IC\(_{50}\) values determined by dose-response curves of ThrRS activity to borrelidin concentration from repeated triplicate experiments with a less than 10% error. The IC\(_{50}\) values we obtained at different ATP or threonine concentrations showed slightly higher values at low ATP or threonine concentrations but essentially the same at higher substrate concentrations, indicating a noncompetitive pattern. Further, we curve fitted data points of IC\(_{50}\) values against substrate concentrations using the equation for a tight binding noncompetitive inhibitor. The plot of IC\(_{50}\) against threonine concentration gave a \(K_i\) value of 4.3 ± 0.2 nM and a \(aK_i\) value of 3.1 ± 0.1 nM. The plot for ATP gave a \(K_i\) value of 4.6 ± 0.2 nM and a \(aK_i\) value of 3.2 ± 0.1 nM. The smaller \(aK_i\) compared with \(K_i\) for both ATP and...
threonine indicated that the conformational changes in E. coli ThrRS upon ATP or threonine binding (23) have some positive influence on borrelidin binding to ThrRS enzymes, although the positive effect is too small to be an uncompetitive pattern. Because borrelidin is a noncompetitive inhibitor with respect to threonine and ATP, borrelidin is not likely to have an overlapping binding site with that of ATP and threonine.

**Screening of ThrRS Mutants Indicates Possible Binding Sites for Borrelidin**—Possible binding sites at the catalytic domain were located by screening a library of randomly mutagenized E. coli thrS for borrelidin-resistant alleles. Eight of ten resistant colonies contained the same Leu489Met thrS allele; another resistant allele is thrS Pro296Ser. Both mutants were subcloned for overexpression as His-tagged proteins, and the purified recombinant enzymes were characterized in vitro. The kinetic data in Table II show that the selected mutant enzymes had a similar $K_m$ value for threonine, an up to 2-fold higher $K_m$ value for ATP, and an up to 2-fold higher $K_m$ value for borrelidin compared with those of the wild type. Therefore, residues at position 489 and 296 are relevant to borrelidin inhibition.


Further, thrS alleles, containing mutations of the predicted contact residues and insertion or deletion constructs of the unique motif three sequence (EGK) of the archaean genre ThrRS, were made by PCR mutagenesis (the mutant changes are described in Fig. 7). After overexpression and purification, the mutant enzymes had good activity. Borrelidin binding to cluster A may cause relocation of Cys-334, resulting in further distortion of the zinc ion coordination and disruption of ThrRS activity (31). As cluster A is very close to the binding sites for threonine, ATP, and the terminal A residue of tRNAThr, substrate binding may also influence cluster A conformation. The fact that other aaRSs do not have such a hydrophobic core in this part of their active site may explain why borrelidin only inhibits ThrRS but not any other aaRSs.

**Comparison of the Putative Borrelidin Binding Region Among ThrRSs**—Alignment of a selection of archaean, bacterial, and eukaryotic ThrRS protein sequences was performed to reveal the extent of sequence conservation of the E. coli ThrRS cluster A residues (Thr-307, His-330, Cys-334, Pro-335, His-337, Leu-489, and Leu-493) position. The Cys-334 residue is conserved in all ThrRS enzymes. The above mentioned cluster A resides (except for Thr-307) are found in the corresponding positions of the human and S. cerevisiae enzyme, both of which are inhibited by borrelidin. Furthermore, Plasmodium ThrRS has the same residues, which may explain its observed antimalarial activity (10). Some bacteria, including some pathogens (e.g. Helicobacter pylori and Mycobacteria), have amino acid replacements in some of the conserved residues in cluster A. The influence of these variations on borrelidin resistance by the corresponding ThrRS enzymes remains to be investigated.

However, the archaean genre ThrRS, found in M. jannaschii,
A. fulgidus, Methanococcus maripaludis, Methanopyrus kandleri, Pyrococci, and Methanosarcineae, contains a different set of amino acids in the corresponding cluster A positions with the exception of the conserved Cys334 (Fig. 10). The absence of cluster A and the resulting borrelidin binding site may explain the exception of the conserved Cys334 (Fig. 10). The absence of cluster A and the resulting borrelidin binding site may explain the exception of the conserved Cys334 (Fig. 10). The absence of cluster A and the resulting borrelidin binding site may explain the exception of the conserved Cys334 (Fig. 10).

**General Discussion**—The work presented here indicates that borrelidin is a novel type of aminoacyl-tRNA synthetase inhibitors. In contrast, most tRNA synthetase inhibitors bind at the active site or bind to the tRNA substrate. For example, indolmycin, a tryptophan analog, inhibits tryptophanyl-tRNA synthetase (4), aminoacyl adenylate sulfamates and sulfonamides inhibit aaRS as mimics of the activated substrate aminoacyl-AMP (33), and oligonucleotide inhibitors mimic tRNA features (34). These inhibitors compete with the substrates for binding to the active site and generally are reversible inhibitors. Mupirocin, a topical antimicrobial agent against methicillin-resistant Staphylococcus aureus, binds to the active site of isoleucyl-tRNA synthetase and inactivates the enzyme by incorrect product shuttling between the synthetic and the editing active site (2, 3). Tobramycin, an aminoglycoside, inhibits acylation substrates. The putative borrelidin binding cluster A (Leu-493, His-307, His-309, and Pro-335) is rendered in space filling mode as an orange sphere; Leu-489 is highlighted in green, and Cys-334 is highlighted in purple. ThrRS is colored as red ribbon. The putative hydrophobic cluster B is (Met-509 and Val-338) is rendered as red ribbon.

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FIG. 10. Protein sequence alignment of the borrelidin binding region in ThrRS. The ▼ indicates the residues of the borrelidin binding cluster A. Strictly conserved residues are indicated by dark shading. ThrRS enzymes are from *S. cerevisiae*, *Homo sapiens*, *Halobacterium sp.*., *E. coli*, *S. solfataricus*, M. jannaschii, and *A. fulgidus*. The last two enzymes are not inhibited by borrelidin.
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