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The Thermophilic, Homohexameric Aminopeptidase of *Borrelia burgdorferi* Is a Member of the M29 Family of Metallopeptidases

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Proteases are implicated in several aspects of the physiology of microorganisms, as well as in host-pathogen interactions. Aminopeptidases are also emerging as novel drug targets in infectious agents. In this study, we have characterized an aminopeptidase from the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. The aminopeptidolytic activity was identified in cell extracts from *B. burgdorferi* by using the substrate leucine-7-amido-4-methylcoumarin. A protein displaying this activity was purified from *B. burgdorferi* by a two-step chromatographic procedure, yielding a ~300-kDa homo-oligomeric enzyme formed by monomers of ~50 kDa. Gel enzymography experiments showed that enzymatic activity depends on the oligomeric structure of the protease but does not involve interchain disulfide bonds. The enzyme was identified by peptide mass fingerprinting as the putative aminopeptidase II of *B. burgdorferi*, encoded by the gene BB0069. It shares significant identity to members of the M29/T family of metallopeptidase, is sensitive to bestatin, has a neutral pH optimum, and displays maximal activity at 60°C. Its activity is 1.75-fold higher at the temperature of the mammalian host than at that of the insect host of the pathogen. The activity of this thermophilic aminopeptidase of *B. burgdorferi* (TAP_{Bb}) depends on Zn^{2+}, and temperatures over 70°C promoted its inactivation through a transition from the hexameric state to the monomeric state. Since *B. burgdorferi* is deficient in pathways for amino acid synthesis, TAP_{Bb} could play a role in supplying required amino acids. Alternatively, the enzyme could be involved in peptide and/or protein processing.

Lyme disease is a multisystemic illness resulting from infection with the spirochete *Borrelia burgdorferi* and related organisms (51). It is most commonly spread to humans by the bite of infected, nymphal-stage hard ticks of the genus *Ixodes*. Lyme disease has been reported in 20 countries in temperate areas of North America, Europe, and Asia; in the United States, reported human cases outnumber those of all other arthropod-borne diseases combined. It represents a significant health problem because of its potentially severe cardiovascular, neurological, and arthritic complications, difficulty in diagnosis and treatment, and high prevalence in some geographic regions. *B. burgdorferi* is an invasive spirochete, as evidenced by its migration from the initial lesion, called erythema migrans, to distant tissues. Overall, the pathogenesis of *B. burgdorferi* appears to be dependent on its ability to colonize, grow, and persist in human tissue for months to years and to elicit tissue damage due to immunopathological reactions. The corresponding factors that may account for these activities include mechanisms for acquisition of required nutrients, motility through mammalian host tissues, shared epitopes with human proteins, and ability to evade the immune system (45, 23, 13).

The type strain *B. burgdorferi* B31 has an unusual genome containing a linear chromosome of 910,725 bp and 22 linear and circular plasmids (16). The small genome size is associated with an apparent absence of genes for synthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides. Thus, *B. burgdorferi* is dependent upon the mammalian or tick host for the provision of amino acids and other required components. An ABC oligopeptide transporter system (*opp*) has been well characterized (32, 54, 55), and genes predicted to encode another oligopeptide ABC transporter and a glycine/betaine transporter have been identified (16) (http://www-biology.ucsd.edu/~msaier/transport/). Otherwise, the mechanisms of amino acid acquisition by *B. burgdorferi* are poorly understood.

Proteases fulfill important roles in both the basic physiology and pathogenesis of many microbial pathogens, including the ability to provide required amino acids, to process proteins and peptides, to facilitate local infection and dissemination through host tissue by degrading extracellular proteins, and to generate factors that are involved in the entry of a parasite into the host cells (4, 9, 29, 47). These activities may also be important among pathogenic spirochetes. The genomes of *B. burgdorferi*, *Treponema pallidum* (the causative agent of syphilis), *Treponema denticola* (involved in periodontal disease), and *Leptospira interrogans* (a cause of leptospirosis) contain many genes homologous to those of virulence-related proteases of other pathogens (16, 17, 43, 50). The prolyl-phenylalanine-specific serine protease dentilisin is involved in the pathogenicity of *T. denticola* (44). A high-molecular-mass protease displaying

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collagenolytic activity was detected in in vitro-cultured *B. burgdorferi* by Grab et al. (22); additionally, Heroldova et al. (24) demonstrated the presence of leucine arylamidase activity in all 13 *B. burgdorferi* sensu lato strains examined, using the API ZYM system. However, these studies were complicated by the presence of serum in the in vitro cultures, which may contribute proteinase activities; also, other groups were not able to detect protease activity in *Borrelia* cultured in vitro (11). Other factors that may contribute to the dissemination and pathogenesis of *B. burgdorferi* in the mammalian host involve the activation or induction of host proteolytic activities. A urokinase-type plasminogen activator binds to the surface of the spirochete, resulting in plasmin generation (10). In addition, host matrix metalloproteinases are induced during *B. burgdorferi* infection and may contribute to tissue pathology (19, 26, 28, 56).

Aminopeptidases (EC 3.4.11) remove N-terminal amino acids from proteins and peptides, are found in animals, plants, and microorganisms, and have several different cellular functions, such as protein maturation and remodeling, hydrolysis of regulatory peptides, antigen processing, and modulation of gene expression. The combined enzymatic activities of both endopeptidases and aminopeptidases mediate hydrolysis of proteins and peptides into free amino acids. Aminopeptidases are considered novel and important pathogen targets for drugs, as evidenced by the relevance of their activities to the life cycle of *Plasmodium* (18, 25, 38) and the African trypanosome (30). In addition, vaccination of sheep with leucyl aminopeptidase from *Fasciola* has induced protection against fascioliasis (40).

In this study, we examined the proteolytic activities present in *B. burgdorferi* B31, utilizing organisms cultured in serum-free medium to obviate the involvement of serum components. We report the identification and characterization of an aminopeptidolytic activity displayed by a thermophilic metalloaminopeptidase of *B. burgdorferi* (TAP_{Bb}). Its enzymatic and biochemical features lead us to consider TAP_{Bb} a member of the thermophilic (T)/M29 family of metallopeptidases. TAP_{Bb} shows restricted enzymatic activity that depends on its oligomeric structure. We postulate that the TAP_{Bb} may play a role in bacterial nutrient supply and/or protein processing.

MATERIALS AND METHODS

Bacteria and preparation of cell extract. *Borrelia burgdorferi* B31 clone 5A3 (39) was cultured at 34°C to mid-log phase in a modified, serum-free form of BSK-II medium (41). Cell extracts were prepared from 100-ml bacterial cultures. The cells were collected by centrifugation (6,000 × *g* for 10 min at 4°C), washed three times in phosphate-buffered saline, lyophilized, and stored at −80°C. The pellet (7.4×10^{10} cells) was resuspended in 7 ml of water containing 1% Triton X-100 and 0.17% lysozyme; the bacteria were immediately disrupted by sonication with six cycles of 30 s each in an ice bath. After removal of the insoluble material by centrifugation (16,000 or 100,000 × *g* for 30 min at 4°C), the supernatant, referred to hereafter as enzyme extract, was used in the assays or stored at −80°C. Protein concentrations in samples were determined as described by Bradford (5).

Assay of proteolytic activity. *B. burgdorferi* protease activity was assayed by using a series of peptidyl fluorogenic substrates (purchased from Sigma Chemical Co.): *N*-glutaryl-Gly-Gly-Phe-4-metoxy-β-naphthylamide, *N*-succinyl-Gly-Pro-7-amido-4-methylcoumarin, *L*-Leu-7-amido-4-methylcoumarin (L-AMC), *N*-carbobenzoxy (CBZ)-Leu-4-metoxy-β-naphthylamide, *L*-Pro-7-amido-4-meth-ylcoumarin, *N*-succinyl-Gly-Pro-Leu-Gly-Pro-7-amido-4-methylcoumarin,*N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin, *N*-CBZ-Gly-Gly-Arg-7-amido-4-methylcoumarin, Gly-Phe-4-metoxy-β-naphthylamide, *N*-CBZ-Phe-Arg-7-amido-4-methylcoumarin, Gly-Pro-7-amido-4-methylcoumarin, *N*-α-CBZ-*L*-Arg-7-amido-4-methylcoumarin, *N*-α-CBZ-Arg-Arg-4-metoxy-β-naphthylamide,*L*-Ala-*L*-Ala-*L*-Phe-Ala-7-amido-4-methylcoumarin, *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin, *N*-succinyl-Ile-Ala-7-amido-4-methylcoumarin, and Gly-Arg-4-metoxy-β-naphthylamide. Enzyme activity was determined by measuring the fluorescence of either 7-amido-4-methylcoumarin (AMC) or 4-metoxy-β-naphthylamide (MNA) released upon hydrolysis of the substrates as described previously (47). Briefly, assays were performed by incubating 1 μl of enzyme extract (3.2 μg of protein) or 30 ng of purified TAP_{Bb}, as specified, for 15 min at the desired temperature in 100 μl of 25 mM Tris-HCl (pH 7.5) (reaction buffer), containing 20 μM fluorogenic substrate. The fluorescence of free AMC or MNA released by enzymatic reaction was recorded in a HITACHI F-2000 spectrofluorimeter as described previously (48). Fluorescence intensities were converted into micromoles of either AMC or MNA by measuring the fluorescence of standard solutions of these fluorogenic groups under the same experimental conditions. Enzymatic activity is expressed in milliunits per milligram, where 1 U represents 1 mmol of released fluorochrome · min^{-1}. In-gel enzymatic activity of either enzyme extract (10 μg) or purified TAP_{Bb} (0.5 μg) on L-AMC was measured on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis (PAGE) at 25°C as described previously (48).

Purification and electrophoretic analysis of the aminopeptidase. *B. burgdorferi* protease, which hydrolyzes L-AMC, was purified from freshly prepared enzyme extract by fast protein liquid chromatography. The enzyme extract (1 ml; 3.2 mg of protein) was buffered with 25 mM Tris-HCl, pH 7.5, and applied to a DEAE-Sepharose CL-6B (Sigma) column (5 by 1 cm) previously equilibrated with the same buffer. The column was washed, and the proteins were eluted with a linear gradient performed in the same buffer from 0.3 to 0.65 M NaCl for 30 min and then with 1.0 M NaCl for 10 min at 0.5-ml/min flow rate. Fractions (2 ml) were collected on ice, and an aliquot of each fraction was assayed with L-AMC. The enzymatically active fractions, which were eluted between 0.46 and 0.52 M NaCl, were pooled and concentrated to 100 μl with a Centricon-100 concentrator (Amicon, Beverly, Calif.) at 4°C. The solution was then subjected to gel permeation chromatography on a Superose-6 HR 10/30 column (Pharmacia) isocratically perfused with 25 mM Tris-HCl, 150 mM NaCl (pH 7.5) at a flow rate of 0.3 ml/min for 80 min. Each 300-μl fraction was immediately stored on ice until the enzyme activity assay. The active fractions were pooled, concentrated as described above, and stored at −20°C. The column was calibrated with bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). The purity and the molecular mass of the purified enzyme were analyzed by SDS–8% PAGE (31) and Coomassie blue staining. The oligomeric structure of the aminopeptidase and the presence of interchain disulfide bonds were evaluated by electrophoresis of either the enzyme extract (20 μg) or purified enzyme (1.0 μg) in either the presence or absence of β-mercaptoethanol (5% [vol/vol]) and with and without prior heating to 100°C for 5 min.

***B. burgdorferi* protease identification by peptide mass fingerprinting.** The purified protein (1.0 μg) was digested with trypsin (Promega, Madison, Wis.) for peptide mass fingerprinting according to the protocol established by Schevchenko et al. (49). The digested sample was applied to a Perkin-Elmer Sciex API 300 electrospray triple-quadrupole mass spectrometer at a flow rate of 0.02 ml/h. Experimentally determined peptide masses were subjected to a protein identity search against the SwissProt database via the program Profound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

Determination of optimal pH and temperature for activity and thermostability of TAP_{Bb}. The optimal pH for TAP_{Bb} activity was assayed as described above in 25 mM Bis-Tris–25 mM Tris-HCl–25 mM borate buffer adjusted to the desired pH. To determine the optimal temperature for TAP_{Bb} activity, the reactions were performed at 20, 25, 30, 40, 50, 60, 70, or 100°C in the reaction buffer. The enzyme thermostability was evaluated by the incubation of the purified protein at the same temperatures for either 10 or 240 min in the reaction buffer, following by an L-AMC hydrolysis assay at 60°C. Molecular organization of TAP_{Bb} preincubated at different temperatures was evaluated by SDS–8% PAGE under nonreducing conditions as described previously (31) and gel permeation chromatography as described above.

Assay of protease inhibition. The inhibition pattern of the enzyme was assayed in reaction buffer using L-AMC as a substrate. Different concentrations of EDTA, 1,10-phenanthroline, bestatin, *L-trans*-epoxysuccinylleucylamido-(4-guanidino) butane (E-64), phenylmethylsulfonyl fluoride (PMSF), *N*-α-tosyl-lysine chloromethyl ketone, leupeptin, pepstatin A, and phosphoramidon were incubated with 30 ng of purified TAP_{Bb} in 100 μl of reaction buffer for 15 min at 25°C before the substrate was added. The enzymatic activity was monitored as described above. All inhibitors were from Sigma.

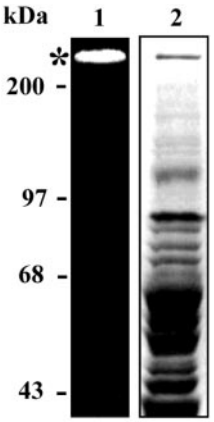

FIG. 1. In-gel leucyl aminopeptidase activity of *B. burgdorferi* protein extract. Enzyme extract, 20 μg, was subjected to SDS–8% PAGE, without previously boiling the sample, at 4°C. The gel was washed and incubated with 20 μM Leu-AMC in reaction buffer at 25°C. The fluorescence of enzymatically released AMC was visualized through UV gel illumination (lane 1, *) prior to gel staining (lane 2).

TAP_{Bb} kinetics and cation dependence. The purified TAP_{Bb} (30 ng) was incubated with different concentrations (1 to 100 μM) of L-AMC, and the enzyme reaction was carried out as described above. Kinetic parameters were determined by hyperbolic regression as described previously (12). All assays were done in triplicate and repeated at least three times. The K_{cat} was calculated by the equation $K_{cat} = V_{max}/[E]_o$, where $[E]_o$ represents active enzyme concentration. The effects of divalent cations on enzymatic activity were assessed by incubating purified TAP_{Bb} in reaction buffer containing either 10 mM EDTA or 250 μM 1,10-phenanthroline for 15 min at 25°C. After extensive dialysis against reaction buffer, L-AMC (final concentration, 20 μM) and $CaCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, or $CoCl_2$ (final concentration, 0.4 mM) were added to the reaction system, followed by 15 min of incubation. Substrate hydrolysis was measured as described above. Controls consisted of enzymatic reactions carried out either in the absence of divalent cations or without EDTA treatment.

RESULTS

***B. burgdorferi* enzyme extract hydrolyzes the protease substrate Leu-AMC.** In preliminary studies, it was determined that enzyme extracts from *B. burgdorferi* grown in Barbour-Stoenner-Kelly medium with rabbit serum produce two major bands of proteolytic activity in SDS-PAGE gelatin, whereas that from bacterium grown in the absence of serum did not (data not shown). This finding is consistent with the acquisition of plasmin and other host proteases by *B. burgdorferi*, as reported previously by other groups (10, 11, 26). To detect proteolytic activity in *B. burgdorferi* itself, we prepared an enzyme extract from organisms grown in a serum-free medium (41) and incubated it with different synthetic fluorogenic substrates. Among all substrates tested (see Materials and Methods), only L-AMC was hydrolyzed by enzyme extract. The calculated specific enzymatic activity was 1,126 ± 108 mU/mg for enzyme extract obtained upon centrifugation at either 16,000 or 100,000 × *g*. This indicates that the enzyme is not associated to membranes. Similar specific enzymatic activity was obtained with enzyme extract prepared with *B. burgdorferi* grown in the presence of serum (data not shown). This proteolytic activity was also assessed by an in-gel procedure that allowed us to estimate the molecular mass of the protease. For this analysis, enzyme extract proteins were separated by SDS-PAGE at 4°C, followed by gel washing for SDS removal. Upon incubation of the gel with L-AMC at pH 7.5, a single fluorescent band corresponding to proteolytic activity was revealed above the 200-kDa molecular weight marker (Fig. 1, lane 1). A protein band comigrating with the enzymatic activity was observed after staining of the same gel (Fig. 1, lane 2).

Purification of the protease and demonstration of its oligomeric form. The protease displaying activity on L-AMC was purified from *B. burgdorferi* by subjecting the enzyme extract to a combination of ion exchange and gel permeation chromatography. Table 1 shows a summary of the purification procedure. The protease activity was eluted from a DEAE-Sepharose CL-6B column at 0.46 to 0.52 M concentrations of NaCl and from a Superose-6 HR 10/30 column as a single peak of activity (data not shown), which indicates that under the condition of this experiment, only one enzyme of *B. burgdorferi* hydrolyzes L-AMC. The last chromatographic step was also used to obtain an estimate of the molecular mass of the enzyme. The enzymatic activity on L-AMC eluted with an apparent molecular mass of 300 kDa (average for four experiments). The same result was obtained when a freshly prepared enzyme extract was applied directly to the column, showing that the ion exchange chromatography did not modify the molecular mass of the protease. The lack of hydrolysis of additional peptidase substrates suggests that the purified enzyme displays narrow activity. The enzyme hydrolyzed L-AMC but not its N-terminal blocked version (*N*-CBZ-L-AMC). Furthermore, no enzymatic activity was observed on other N-terminal free substrates, such as Pro-AMC, Gly-Phe-MNA, Gly-Pro-AMC, and Gly-Arg-MNA. These data indicate that the enzyme is an aminopeptidase that preferentially removes leucine from N termini of peptides and/or proteins.

The electrophoretic profile of each step of the purification procedure is shown in Fig. 2. Although the protease was obtained with high purity (Fig. 2, lane 3), in some preparations an additional protein band of about 50 kDa was seen when electrophoresis was carried out under reducing conditions without previous boiling of the sample (Fig. 2, lane 4). However, unlike the higher-molecular-mass band, the 50-kDa band did not hydrolyze the substrate L-AMC, as evidenced by in-gel enzymatic activity experiment (Fig. 2, lane 5). To investigate whether the two bands corresponded to different oligomeric states of the same protein and whether the temperature could interfere with that pattern, the purified protease was boiled for 5 min before SDS-PAGE analysis under reducing conditions (Fig. 2, lane 6). Upon staining of the gel, only the 50-kDa band was revealed. These data suggested that the active 300-kDa protease is an oligomeric protein formed by monomers of about 50 kDa.

To investigate whether interchain disulfide bonds partici-

TABLE 1. Purification of aminopeptidase from *B. burgdorferi*

Procedure	Sp act[a]	Total activity[b]	% Yield	Purification factor
Enzyme extract	6.0×10^{-2}	20.5×10^{-2}	100	1
DEAE-Sepharose	15.0×10^{-2}	18.0×10^{-2}	88	3
Superose 6	102.9×10^{-2}	15.4×10^{-2}	73	32

[a] Specific activity: nanomoles of AMC released per minute per milligram of protein.
[b] Total activity: specific activity × total protein.

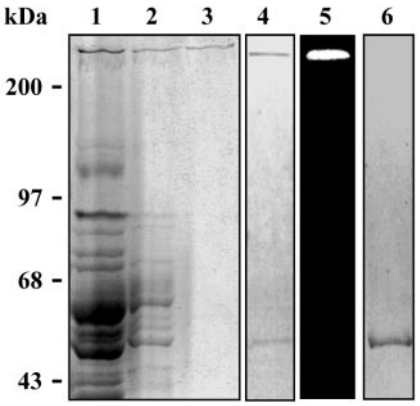

FIG. 2. Electrophoretic analysis of purified aminopeptidase from *B. burgdorferi.* Samples from crude enzyme extract (10 μg of protein; lane 1), DEAE-Sepharose column fractions (4 μg; lane 2), or Superose-6 column fractions (0.5 μg; lanes 3 to 6) were subjected to SDS–8% PAGE. Electrophoresis took place at 4°C under reducing conditions either with previous boiling of the sample (lane 6) or not (lanes 1 to 5). The gel in lane 4 was previously incubated with Leu-AMC (lane 5) for the in-gel activity assay, as described in the Materials and Methods section. Gels were silver stained.

pated in the arrangement of the oligomer, we subjected either enzyme extract or purified enzyme, previously boiled or not, to SDS–8% PAGE in the presence or absence of β-mercaptoethanol (Fig. 3). The reducing agent did not affect the electrophoretic migration pattern of nonpurified (lanes 1 and 2) and purified (lanes 3 and 4) protease. In contrast, the 300-kDa oligomer was observed in the gels only where the samples have not been previously boiled (lanes 2 and 4), while its 50-kDa monomer was revealed upon sample boiling (lanes 1 and 3). Since high temperature induced the monomerization of the 300-kDa oligomer independently of the presence of β-mercaptoethanol, we conclude that interchain disulfide bonds do not take part in the stabilization of the active protease oligomer.

Molecular identification of the purified protease. To identify the purified enzyme mediating L-AMC hydrolysis, the enzyme was digested with trypsin and the resulting peptides were submitted to mass spectrometry for peptide mass fingerprinting. Mass values obtained for the detected peptides were compared to those theoretically deduced from sequences deposited in the database. Eight peptides showed very close mass matches to peptides obtained by theoretical digestion of a predicted aminopeptidase of *B. burgdorferi* (Table 2), encoded by the gene BB0069 (16) (SWISS-PROT/TrEMBL accession no. O51096). This result supports our experimental data showing that the purified enzyme mediates aminopeptidolytic activity. The BB0069 gene comprises an open reading frame that encodes a 412-amino-acid protein with a calculated molecular mass of 46,798 Da and no predicted peptide signal. Based on sequence homology, BB0069 is unrelated to three other predicted aminopeptidases in the *B. burgdorferi* genome: BB0105 (methionine aminopeptidase); BB0366 (YscI or aminopeptidase I; previously reported by Wallich et al. as APEA_BORBU [SWISS-PROT/TrEMBL]); and BB0627 (PepX; vacuolar X-prolyl dipeptidyl aminopeptidase I). Interestingly, *Treponema denticola* 35405 contains a homolog of BB0069 (TDE 2337; 34% identity, 57% similarity; Fig. 4A), but no homologs are present in the *T. pallidum* or *L. interrogans* genomes. Although BB0069

TABLE 2. Identification of *B. burgdorferi* protease by peptide mass fingerprinting

Tryptic peptide mass (Da)		Identified amino acid sequence
Experimental data[a] (in-gel digestion)	*B. burgdorferi* aminopeptidase (in silico digestion)	
671.680	671.795	^{16}GINLQK^{21}
1,183.352	1,183.261	^{288}VVDFGCDDEK^{297}
1,314.408	1,314.414	^{159}EGSQTLEEFFK^{169}
1,919.089	1,919.249	^{199}TLNELNLEKVIFKTEK^{214}
2,890.105	2,890.220	^{236}GTEIEFNANMPTEEVFTTP NYKKTCK^{260}
1,919.152	1,919.249	^{199}TLNELNLEKVIFKTEK^{214}
2,187.480	2,187.656	^{39}ILAKKAYEHAKYVKLNIK^{57}
1,200.348	1,200.444	^{58}DIDILKSRLK^{67}

[a] Masses are MH+ monoisotopic, with accuracy of 0.1 to 0.2 Da. The enzyme was digested with trypsin, and masses of resulting peptides were determined by electrospray ionization–mass spectrometry and compared to theoretical ones produced by in silico digestion of proteins found in the database (SwissProt).

is within a possible multicistronic operon (BB0069-BB0073), none of the genes in this region are clustered in other organisms or have related predicted functions (data not shown).

Multiple amino acid sequence alignments confirmed that this *B. burgdorferi* aminopeptidase is a member of the thermophilic metalloaminopeptidase (M29) family of metallopeptidases, also known as the T (thermophilic) family (42). It shared 32 to 36% identity to other members of M29 family, including assigned and unassigned aminopeptidases (Fig. 4A). Sequence alignments also revealed that the C-terminal portion is the most conserved region in this family, reaching 44% identity and 63% similarity between *B. burgdorferi* and *Geobacillus stearothermophilus*. Although identity among members of the M29 family and those of the M17, leucyl aminopeptidase fam-

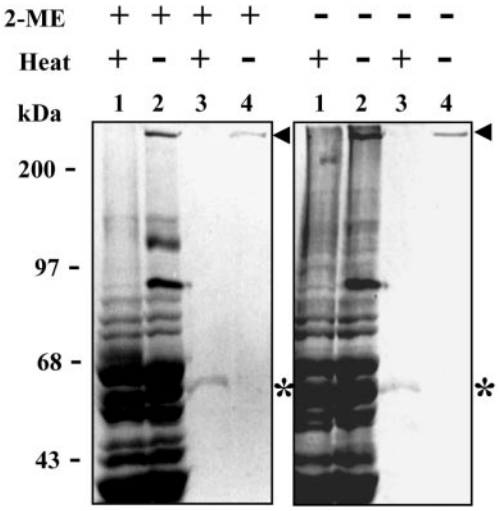

FIG. 3. The oligomeric structure of TAP_{Bb} does not contain interchain disulfide bonds. Either 20 μg of enzyme extract (lanes 1 and 2) or 2 μg of purified TAP_{Bb} (lanes 3 and 4) was subjected to SDS–8% PAGE under reducing (2-ME +) or nonreducing (2-ME −) conditions. Samples in lanes 1 and 3 were heated (+), while those in lanes 2 and 4 were not (−). The gels were stained with Coomassie blue. Arrowheads and asterisks indicate positions of ~300- and ~50-kDa bands, respectively.

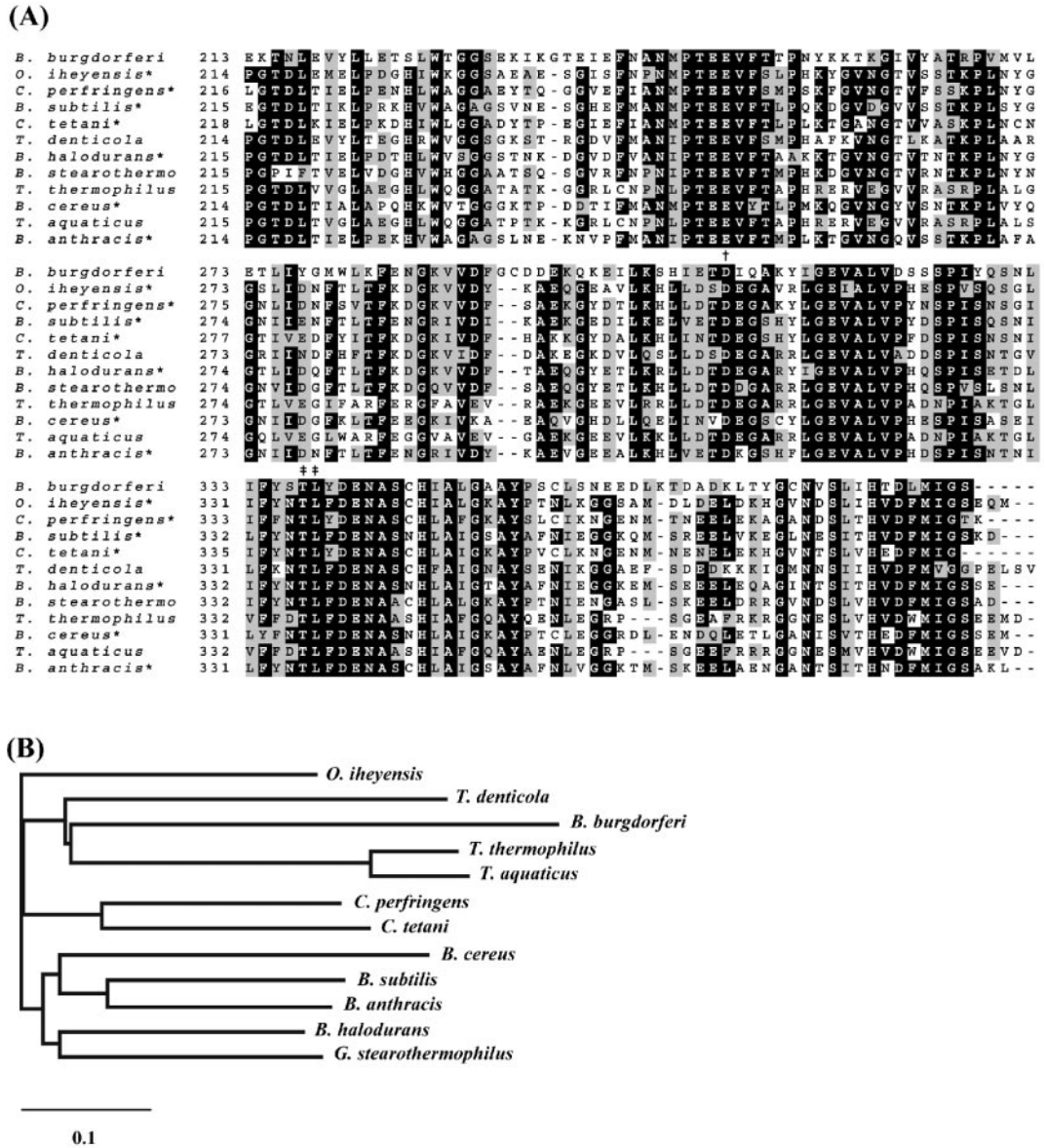

FIG. 4. Sequence comparison and relationship of TAP_{Bb} to other members of the M29 family of metallopeptidases. (A) Multiple sequence alignment of C-terminal-portion amino acid sequences of aminopeptidases. The putative zinc binding (†) and bestatin ligation residues (‡) are represented. Sequences were obtained from the GenBank/EBI database under the following accession numbers: AAC66461 (*Borrelia burgdorferi*), BAC14998 (*Oceanobacillus iheyensis*), TDE 2337 (*Treponema denticola*), P24828 (*Geobacillus stearothermophilus*), NP_389328 (*Bacillus subtilis*), NP_562913 (*Clostridium perfringens*), NP_782566 (*Clostridium tetani*), NP_243111 (*Bacillus halodurans*), NP_654259 (*Bacillus anthracis*), NP_831585 (*Bacillus cereus*), P42778 (*Thermus thermophilus*), and P23341 (*Thermus aquaticus*). (B) Phylogenetic relationship of members of the aminopeptidase M29 family. A phylogram was generated after alignment of the full-length amino acid sequences of the 11 enzymes, using CLUSTAL W software with a PAM250-weight table set with the parameters Ktuple = 1, opening penalty = 3, and gap extension = 5. The scale at the bottom represents the number of amino acid substitutions per site. Asterisks indicate functionally defined enzymes.

ily is almost absent (data not shown), sequence alignments suggested conservation of zinc and bestatin binding residues (Fig. 4) (7, 36). In contrast, sequences of members of the M29 family do not comprise the signature NTDAEGRL sequence of the M17 family (7). These data suggest that TAP_{Bb} is unrelated to the M17 family, in spite of displaying leucyl aminopeptidase activity. Three-dimensional structures and the active-site residues are not known for any members of the M29 family.

***B. burgdorferi* aminopeptidase is a neutral, thermophilic, and thermostable enzyme.** The effect of pH on hydrolysis of L-AMC by the aminopeptidase was determined. It was shown that the enzyme has a strong dependence on neutral pH. Maximal specific activity for the purified enzyme was observed at pH 7.5 (data not shown). At pHs 6.0 and 9.0, the measured enzymatic activities were 20 and 50% of that recorded at pH 7.5. The optimum temperature for aminopeptidase activity on the L-AMC substrate was shown to be 60°C (Fig. 5), and

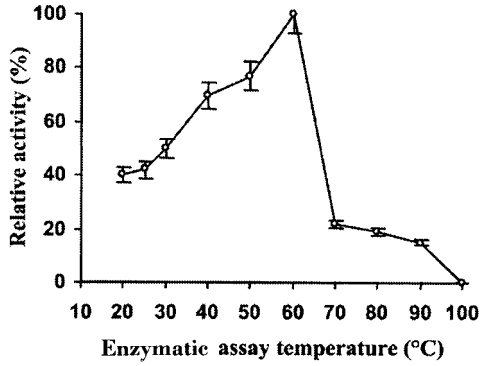

FIG. 5. Optimal temperature for TAP_{Bb} activity. Purified aminopeptidase was incubated with Leu-AMC in reaction buffer at different temperatures for 15 min. Enzymatically released AMC was quantitated as described in Materials and Methods. Results are expressed as the percent activity relative to the values obtained at 60°C. Assays were carried out in triplicate, and results represent means for three separate experiments. Standard deviations were less than 8%.

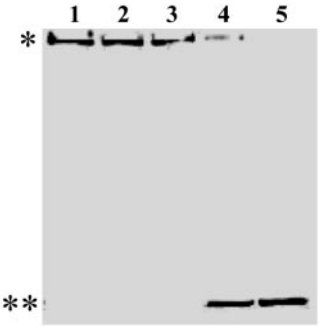

FIG. 6. Electrophoretic profile of TAP_{Bb} upon preincubation at different temperatures. Purified protein was incubated at 37 (lane 1), 50 (lane 2), 60 (lane 3), 70 (lane 4), or 100°C (lane 5) for 10 min in the reaction buffer, following by SDS–8% PAGE analysis under nonreducing conditions. The gel was silver stained. Oligomeric (*) and monomeric (**) states of TAP_{Bb} are indicated.

we thus named the enzyme thermophilic aminopeptidase of *B. burgdorferi* (TAP_{Bb}) and named its gene thermophilic aminopeptidase A (*tapA*). At 20°C the enzymatic activity corresponded to 40% of that measured at 60°C, while at 70°C the enzyme retained 25% of its activity on the substrate. Interestingly, at 40°C the activity of the enzyme was about 1.75 times higher than that measured at 20 to 25°C, the body temperature of the insect. This might suggest that the enzyme activity is regulated depending on the *B. burgdorferi* host. To determine the thermostability of TAP_{Bb}, we assayed substrate hydrolysis by the enzyme at 60°C after its preincubation at different temperatures for either 10 or 240 min (Table 3). The data show that its activity was not significantly modified after a preincubation at 60°C during 240 min. Its preincubation for 10 min at 70°C resulted in significant loss of activity.

TAP_{Bb} enzymatic activity depends on its oligomeric form. Since preincubation of TAP_{Bb} for 10 min at 70°C resulted in significant loss of its activity, we asked whether this enzymatic inactivation was due to monomerization of the oligomer. To address this question, we first incubated TAP_{Bb} at different temperatures for 10 min, following by SDS-PAGE analysis. Temperatures up to 60°C did not modify the oligomeric state of the protease, since it migrated as a single high-molecular-mass band in the gel (Fig. 6, lanes 1 to 3). However, upon preincubation at 70°C, the enzyme appeared mostly in its monomeric form, a protein band of 50 kDa (Fig. 6, lane 4). This correlated well with enzymatic activity measured under the same conditions (Table 3). In addition, enzymatic activity was no longer detected upon preincubation at 100°C, most likely due to entire monomerization of the oligomer (Fig. 6, lane 5). Secondly, we evaluated the molecular mass of the active protease, previously subjected to different temperatures, by gel permeation chromatography. Hydrolysis of L-AMC coeluted with the oligomer up to 60°C preincubation, while higher temperatures progressively induced the appearance of the inactive 50-kDa monomer (data not shown). These data indicate that the TAP_{Bb} activity depends on its oligomeric form.

TAP_{Bb} is a metalloaminopeptidase dependent on zinc. TAP_{Bb} hydrolytic activity towards L-AMC was completely inhibited by 250 μM 1,10-phenanthroline or 100 μM bestatin, while 10 mM EDTA inactivated only 50% of the activity (Table 4). Its activity was not sensitive to E-64, PMSF, *N*-α-tosyllysine chloromethyl ketone, leupeptin, or pepstatin A. The activity of TAP_{Bb} previously inhibited by either 10 mM EDTA or 250 μM 1,10-phenanthroline was fully restored with 0.4 mM Zn^{2+} but not with Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, or Co^{2+} (Table 4). This indicates that TAP_{Bb} is a zinc-dependent metalloaminopeptidase.

TABLE 3. TAP_{Bb} is thermostable[a]

Preincubation temp of enzyme (°C)	Enzymatic activity (% of control)	
	10-min preincubation	240-min preincubation
25	100	98
37	101	99
50	105	95
60	102	87.5
70	25	9
100	0	0

[a] Purified enzyme was preincubated at different temperatures for 10 or 240 min, and then its activity on Leu-AMC was measured at 60°C. Control consisted of enzymatic activity assay without previous incubation of enzyme.

TABLE 4. Inhibition pattern and cation dependence of TAP_{Bb}[a]

Concn and inhibitor	TAP_{Bb} activity (% of control) with:					
	No cation	Zn^{2+}	Mn^{2+}	Ca^{2+}	Cu^{2+}	Co^{2+}
250 μM 1,10-phenanthroline	0.00	98	0.00	0.20	0.70	0.20
10 mM EDTA	50.0	93	47	52.5	49.3	53.1
100 μM bestatin	0.00	0.00	0.00	0.00	0.00	0.00
2 mM PMSF	96.4	ND[b]	ND	ND	ND	ND
15 μM E-64	96.7	ND	ND	ND	ND	ND
15 μM TLCK	98.2	ND	ND	ND	ND	ND
200 μM pepstatin A	102.5	ND	ND	ND	ND	ND
200 μM leupeptin	95.8	ND	ND	ND	ND	ND

[a] Purified TAP_{Bb} was incubated with inhibitor before the addition of Leu-AMC. Enzyme incubated with 1,10-phenanthroline or EDTA was dialyzed against reaction buffer prior to incubation with the substrate and 0.4 mM $CaCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, or $CoCl_2$. Reactions took place at 60°C, and results represent means from three independent experiments carried out in triplicate. Standard deviations were less than 10%.

[b] ND, not determined.

TAP_{Bb} kinetic parameters. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of purified TAP_{Bb} were determined by using the hyperbolic regression method with L-AMC as a substrate. The aminopeptidase has a K_m value of 45.1 ± 1.9 μM. Its calculated catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were 8.03 ± 0.71 S^{-1} and 17.8×10^{-2} $\mu M^{-1} \cdot S^{-1}$, respectively.

DISCUSSION

In this study, we identified the product of BB0069 as a thermophilic aminopeptidase and named it TAP_{Bb} to indicate this activity. TAP_{Bb} hydrolyzed the fluorogenic substrate L-AMC in a specific manner, as evidenced by the absence of detectable hydrolysis of any other substrate used. None of the other proteolytic substrates were hydrolyzed by the *B. burgdorferi* cytosolic extract under the conditions examined. These results suggest that proteolytic activity of *B. burgdorferi* is highly restrictive, which may explain why this invasive bacterium employs mammalian host proteases with broad specificity for its own benefit (10, 11, 26).

Secreted bacterial aminopeptidases are monomeric, while the intracellular enzymes are either monomeric or multimeric, comprising one, two, four, or six monomers (21). None of the aminopeptidases of the M29 family described to date is hexameric: aminopeptidase T of *Thermus aquaticus* and *Bacillus stearothermophilus* are homodimeric enzymes (3, 37), and PepS of *Streptococcus thermophilus* is monomeric (14). The hexameric quaternary structure of TAP_{Bb} is required for activity, but the mechanism of this structural dependence is not currently known. The hexameric structure implies the binding of complementary hydrophobic regions between subunits and reduction of the molecule surface with the medium, thus limiting the amount of water required to stabilize these proteins (20). Disulfide bridges do not take part in the oligomeric assembly of TAP_{Bb}, because monomerization did not depend on the presence of a reducing agent. This is in agreement with observations for other members of the M29 family (3, 37) as well as other aminopeptidases, such as leucyl aminopeptidase of bovine lens and aminopeptidase A of *Escherichia coli* (8, 53). The correlation between the multimeric structure and activity of TAP_{Bb} indicates either that the active site is formed at the subunit junctions or that subunit interactions stabilize the active form of the monomers; additional experimentation will be needed to resolve this question.

TAP_{Bb} is the first M29 aminopeptidase characterized from any pathogenic microorganism. To date, in addition to bacteria, genes encoding M29 aminopeptidases have been identified in archea (27) and plants (EMBL AJ004922). However, only aminopeptidases from *T. aquaticus*, *Thermus thermophilus*, *G. stearothermophilus*, and *S. thermophilus* have been conclusively characterized as members of this family (14, 36, 42). Among the features that justify this classification are thermophilicity, high conservation of C termini, and no significant identity with other known aminopeptidases. In addition to considerable sequence identity with members of the M29 family, TAP_{Bb} is thermophilic. Its optimal temperature for activity was observed at 60°C, identical to that for the aminopeptidases of *G. stearothermophilus* and *S. thermophilus* (3, 14) and similar to that determined for *T. aquaticus* aminopeptidase (70°C) (35). In contrast to these microorganisms, *B. burgdorferi* is not thermophilic. However, it is likely that bacteria originated from hyperthermophilic organisms (1, 6). Forterre (15) postulated that the mesophilic prokaryotes existing today must have evolved through a gradual adaptation of thermophilic enzymes to lower temperature optima. Spirochetes have been positioned as one of the most primitive bacterial phyla in the universal evolutionary tree (6). It is of interest that the free-living spirochete *Spirochaeta thermophila* is thermophilic. Thus, the presence of a thermophilic enzyme in *B. burgdorferi* may represent an evolutionary holdover from a thermophilic ancestor. The thermophilic properties of TAP_{Bb} could alter other properties of the enzyme and hence result in a survival advantage, a subject that requires further investigation.

In members of the M29 family characterized previously, the thermostability and temperature optima of the aminopeptidase are higher in the more thermophilic bacteria. For example, the *S. thermophilus* aminopeptidase PepS retains 59% of its activity after incubation at 50°C for 20 min (14). Although not produced by a thermophilic bacterium, TAP_{Bb} is even more stable than PepS; it maintained 87.5% of its activity after 240 min at 60°C. The hexameric nature of TAP_{Bb} may increase its thermostability and thus represent an adaptive advantage relative to monomeric counterparts as PepS. Other highly thermostable enzymes, such as methylthioadenosine phosphorylase of *Sulfolobus solfataricus* (2) and peptidase B of *Salmonella enterica* (34), are also hexameric proteins.

Bacterial aminopeptidases are subdivided into three main catalytic groups based on their sensitivity to inhibitors: metalloaminopeptidases, whose activity is inhibited by chelating agents, such as EDTA, 1,10-phenanthroline, and bestatin; serine aminopeptidases that are sensitive to PMSF; and cysteine aminopeptidases, those susceptible to a broad range of agents, such as Hg^{2+}, E-64, and iodoacetamide (21). Our data indicate that TAP_{Bb} is a metalloaminopeptidase and Zn^{2+} is its likely cofactor. Although metalloaminopeptidases exhibit a broad range of metal ion dependence, Zn^{2+} is the most frequently associated cation (42). Members of the M29 family from *T. aquaticus* and *G. stearothermophilus* have Co^{2+} and Mg^{2+} as cofactors (35, 52). The cofactor for another characterized member of the M29 family, PepS, is unknown; however, it is unlikely to be Zn^{2+} or Co^{2+}, since low concentrations of these cations inactivate the enzyme (14). Therefore, TAP_{Bb} seems to be the first described member of the M29 family to employ Zn^{2+} to activate the water molecule during catalysis. Among the known metal ligands of metalloproteases, Asp is conserved in TAP_{Bb}. It has been proposed that two phylogenetically unrelated subgroups of Zn^{2+} aminopeptidases, the largest group of metalloproteases, may be distinguished (21). The first comprises aminopeptidases, such as PepN of *E. coli*, that display the divalent cation binding motif HExxH, and the second subgroup comprises aminopeptidases that show peptide sequence similarities with bovine lens leucine aminopeptidase. TAP_{Bb} appears to be unrelated to these subgroups of Zn^{2+} aminopeptidases, since its peptide sequence shows neither the HExxH motif nor considerable similarities with any other Zn^{2+}-requiring aminopeptidases.

Although functional properties are unknown for any member of the M29 family, aminopeptidases have been implicated in several physiological roles, such as antigen processing, nu-

tritional supply, degradation of proteins and peptides of both endogenous and exogenous origin, protein maturation, bacterial sensitivity to antibiotics, and stability of plasmids (33). In this study, TAP_{Bb} comprised the entire leucine aminopeptidase activity in *B. burgdorferi* extracts. Thus, it most likely plays an important role in protein and peptide processing as well as in leucine recycling. *B. burgdorferi* lacks genes coding for amino acid biosynthesis enzymes, and leucine transporters have not been identified for this pathogen (16, 46). An analysis of codon usage indicated that Leu content is essentially equivalent in *B. burgdorferi*, *T. pallidum*, and *E. coli* (10.11, 10.34, and 10.6%, respectively). Also, Leu does not appear to be more frequent than expected at the penultimate N-terminal residue position (which would be susceptible to aminopeptidase action upon removal of the N-terminal *N*-formyl methionine) (data not shown). Therefore, it is unclear why *B. burgdorferi* would require a Leu-specific aminopeptidase. If a biosynthetic pathway is indeed lacking, leucine must be acquired by the bacterium through hydrolysis of host and endogenous proteins. It thus appears that inactivation of TAP_{Bb} by specific inhibitors or through gene disruption would reveal its functional role and consequently its importance in *B. burgdorferi* physiology and pathogenesis.

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