Crystal structure of a Class A β-lactamase from *Nocardia cyriacigeorgica*

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Abstract

Nocardia are gram-positive bacteria from the actinobacterial class. Some *Nocardia* species can infect humans and are usually considered opportunist pathogens as they often infect immunocompromised patients. Albeit, their clinical incidence is low, many *Nocardia* species are nowadays considered emerging pathogens. Primary sites of infection of *Nocardia* are the skin or the lungs but dissemination to other body parts is very frequent. These disseminated infections are very difficult to treat and thus, tackled with multiple classes of antibiotics, on top of the traditional treatment targeting the folate pathway. ß-lactams are often included in the regimen but many *Nocardia* species present moderate or strong resistance to some of this drug class. Genomics, microbiological, and biochemical studies have reported the presence of class A ß-lactamases (ABL) in a handful of *Nocardia* species but no structural investigation of *Nocardia* ß-lactamases has been performed yet. In this study, we report the expression, purification, and preliminary biochemical characterization of the ABL from a *Nocardia cyriacigeorgica* (Bla_{Ncyr}) clinical strain. We described, as well, the crystallization and the very high-resolution crystal structure of Bla_{Ncyr}. The protein sequence and structural analysis demonstrate that Bla_{Ncyr} belongs to ß-lactamase of class A1 and attest to very high conservation between ABL from other human pathogenic *Nocardia* species. In addition, the presence of one molecule of citrate tightly bound in the catalytic site of the enzyme is described. This structure may provide a solid basis for future drug development to specifically target *Nocardia* ß-lactamases.

1 Introduction

Nocardiosis is an infectious disease caused by environmental bacteria from the *Nocardia* gender. *Nocardia* are Gram-positive bacteria from the actinomycetes class. To date, about 130 species of *Nocardia* have been reported (https://www.bacterio.net/genus/nocardia) of which about 40 species are human pathogens and clinically relevant (Mehta and Shamoo, 2020). *Nocardia* are usually opportunistic bacteria that often trigger pulmonary infections. They also frequently disseminate to other organs skins, joints, or the brain. Central nervous infections are very acute forms of nocardiosis as the mortality rate ranges from 40 to 80% (Cattaneo et al., 2013; McNeil and Brown, 1994; Wilson, 2012). The incidence of *Nocardia* infections is rather low but certainly underestimated as identification of *Nocardia* species has been impaired by technical aspects, as it is mainly based on gene sequencing. Powerful methods such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Hodille et al., 2023) will likely enable better identification and may help apprehend the real incidence of this emerging class of bacteria.

For long the reference treatment of nocardiosis, whatever the species, was based on a combination of two antibiotics, trimethoprim (TMP) and sulfamethoxazole (SMX), targeting the folic acid pathway (Margalit et al., 2021). Nonetheless, treating nocardiosis with this regimen remains very challenging and is often punctuated by treatment failure and/or relapse. This is mainly due to the high prevalence of drug resistance strains to TMP-SMX (Uhde et al., 2010). It is extremely difficult to treat the disseminated forms of *Nocardia* particularly those of the central nervous system. Treatment success is also impaired by the difficulty of choosing and adapting the right drug regimen since there is a large heterogeneity of drug susceptibility and drug resistance between the different human pathogenic *Nocardia* species (Hershko et al., 2023; Lebeaux et al., 2019).

To circumvent these issues, the current recommendation is therefore a combination of different antibiotic classes. On top of aminoglycosides and oxazolidinones, β-lactams are often used to treat nocardiosis. β-lactams resistance has however been reported for *Nocardia* with strong variations from one species to another. As stated in a retrospective study while about 80% of *Nocardia farcinica* are resistant to the β-lactam cefotaxime only 5% of the *Nocardia cyriacigeorgica* strains are (Lebeaux et al., 2019). β-lactamases are enzymes capable of

hydrolyzing β-lactam antibiotics and are classified into four classes according to the Ambler nomenclature (Ambler et al., 1991; Bush, 2013). Classes A, C, and D are serine hydrolases while class B are metallo-β-lactamases that accommodate one or two zinc ions in their active site (Bahr et al., 2021). It is not yet fully documented if *Nocardia* species possess all four β-lactamases classes, however, they certainly widely harbor class A β-lactamases (ABL) (Valdezate et al., 2015). ABL are usually capable of hydrolyzing a wide range of β-lactams and have been found or can be found in the genomes of most *Nocardia* species. Only three studies have described the biochemical characterization of ABL, notably from *Nocardia farcinica* (Laurent et al., 1999; Lebeaux et al., 2019) and *Nocardia asteroides* (Poirel et al., 2001).

To date, there is no report of any three-dimensional structure of ABL from *Nocardia*, such data could be helpful to fully apprehend the difference of substrate specificity and inhibitors efficacies observed between ABL from different *Nocardia* species (Laurent et al., 1999; Lebeaux et al., 2020; Poirel et al., 2001). To fill this gap, we describe in this study the biochemical and structural characterization of a class A Bla from *Nocardia cyriacigeorgica* (Bla_{Ncyr}) one of the most encountered pathogenic human *Nocardia* species and for which ß-lactams resistance has been reported (Zhao et al., 2017).

2 Materials and methods

2.1 Gene amplification and cloning

Genomic DNA extracted from a *N. cyriacigeorgica* clinical isolate responsible for pulmonary nocardiosis was used as a template for polymerase chain reaction PCR with primers 5'-gatatgcaccacggcctgca-3' and 5'-acggcgacgaagaagcgga-3' by using Invitrogen™ Platinum SuperFi II DNA Polymerase (Thermo Fisher Scientific, Illkirch, France). A second PCR was performed on the aforementioned amplicon to amplify the truncated version of *Bla* with the following forward 5'-ggtaccgagaacctgtacttccagggttcggccgtggccgatccccggttcgccgcactggaaacg-3'and reverse primer 5'-gtggtgctcgagctaaccgagcacgtcgacgaccgtcctggtcgcgtcggc-3'. The purified PCR product was treated with DreamTaq polymerase to add overhanging A in 3' and cloned into the Champion™ pET SUMO (Invitrogen) following manufacturer instructions and correction insertion of the DNA fragment was verified by sequencing.

2.2 Protein Expression and Purification

The pET-SUMO::*Bla*$_{Ncyr}$ plasmid was transformed into the *E. coli* BL21 strain resistant to phage T1 (New England Biolabs) harboring the pRARE2 plasmid and plated onto LB+Agar plates supplemented with kanamycin (Kan) (50 □g.mL^{-1}) and chloramphenicol (Cam) (30 □g.mL^{-1}). An overnight (ON) preculture was used to inoculate 2 flasks containing each 3L of LB with Kan and Cam. When the cells reached the exponential phase, they were placed into ice for 40 min and protein expression was induced with 1 mM IPTG, and the culture was placed at 18□C ON. Cells were harvested by centrifugation for 15 min at 6000 *g* and resuspended in 80 mL of 50 mM Tris pH 8, 0.4 M NaCl, 5 mM β-mercaptoethanol, 1 mM benzamidine, 10 % glycerol, and 20 mM Imidazole. Bacteria were lysed by sonication and centrifuged for 40 min at 27000 *g*. The clarified extract was loaded onto three gravity columns containing each 2 mL of Ni-NTA Sepharose beads (Cytiva) at 4□C. The columns were then washed with 25 mL of 50 mM Tris pH8, 1 M NaCl, 5 mM β-mercaptoethanol, and 10% glycerol and the proteins were eluted with a total of 30 mL of buffer 50 mM Tris pH8, 0.2 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 200 mM Imidazole. At this stage about 230 mg of protein were recovered and the tag was cleaved by adding 4 mg of Tobacco Etch Virus (TEV) protease in a dialysis bag and dialyzed ON at 4□C against 50 mM Tris pH8, 0.2 M NaCl, 5 mM β-mercaptoethanol and 10% glycerol. A second nickel affinity chromatography was performed to separate the cleaved Bla$_{Ncyr}$ from the tags and the Histagged-TEV protease. To do so, 2 columns with 3 mL beads each were equilibrated with the dialysis buffer and the samples were loaded three times. The columns were washed with 20 mL of dialysis buffer and 25 mL of 50 mM Tris pH 8, 1 M NaCl, 5 mM β-mercaptoethanol and 10% glycerol. About 200 mg of Bla$_{Ncyr}$ protein were recovered in the flow-through and the first wash step, concentrated to 5 mg.mL^{-1} and flash frozen in liquid nitrogen. For biochemical and crystallization purposes the protein was further purified by size-exclusion chromatography (SEC) on a Superdex 75 Increase 10/300 GL column (Cytiva) on which 5 mg of protein were injected and eluted with the following buffer: 20 mM Tris pH8, 0.2 M NaCl, 0.5 mM β-mercaptoethanol and 5% glycerol.

2.3 Assessment of Bla$_{Ncyr}$ oligomeric state

The calibration curve was performed on a Superdex 75 Increase 10/300 GL column possessing a total volume (Vt) of 23.56 mL connected to an Äkta Go (Cytiva). The proteins were

eluted in 20 mM Tris pH8, 0.2 M NaCl and 0.5 mM β-mercaptoethanol. A mixture of 600 µg of bovine serum albumin, 200 µg of anhydrase carbonic, 190 µg of cytochrome C and 400 µg of bovine aprotinin were injected simultaneously and eluted at a flow-rate of 0.3 $ml.min^{-1}$. Bla_{Ncyr} (300 µg) was injected independently and thyroglobulin (400 µg) was used to determine the void volume Vo. The volume of injection for each sample was 500 µL. The partition coefficient of each protein K_{av} was determined as follows: K_{av}=(Vt-Vo)/(Ve-Vo) where Ve indicates the volume of elution of each protein. The calibration curve was obtained by plotting the *K*av against the Log of the molecular weight (M.W.)

2.4 Crystallization

Crystals were obtained in sitting drops using the Swissci MRC MAXI plate mixing 1□L of protein solution concentrated to 14 $mg.mL^{-1}$ with 1□L of reservoir solution made of 0.1 M citric acid pH3.5, 10 % PEG 6000 and 6% ethylene glycol and equilibrated against a reservoir volume of 200 □L at 18□C. Crystals were cryoprotected by the addition of 4 □L of a solution made of 0.1 M citric acid pH3.5, 14 % PEG 6000, and 20 % ethylene glycol, on top of the crystallization drop and incubated for 16 hours before being cryocooled in liquid nitrogen.

2.5 X-ray data collection, structure determination, and refinement

Data collection was performed on the PXI-X06SA beamline at the Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland. A total of 800 images were collected at an oscillation range of 0.2□, a crystal-to-detector distance of 145 mm, and a X-ray wavelength of 0.99 Å. Data were processed and scaled with the XDS package (Kabsch, 2010). The phase problem was solved by molecular replacement using Phaser (McCoy, 2007) and the Phenix package (Liebschner et al., 2019), and an AlphaFold model (Jumper et al., 2021) of Bla_{Ncyr} as a search template. The structure was further manually rebuilt with Coot (Casañal et al., 2020) and refined with the Phenix package (Liebschner et al., 2019). Statistical values given in Tables 3 and 4 were calculated with aimless from the *CCP*4 suite (Agirre et al., 2023) and Phenix (Liebschner et al., 2019).

2.6 Activity assays

The Bla_{Ncyr} β-lactamase assay was performed in 96 wells plates and a reaction volume of 100 □L in 1x phosphate saline (PBS) buffer pH 7 and the presence of 0.2 mM nitrocefin. Reactions

were initiated by addition of 2, 4, or 8 nM of enzyme or 8 nM of enzyme incubated with 500 □M of avibactam for 30 min. A control reaction was performed with all components but the enzyme. The optical density at 486 nm was recorded for 75 minutes with data points acquired every 1 min on a Tecan Infinite® 200 PRO M Plex plate reader.

3 Results and discussion

3.1 Gene amplification, construct design and protein expression

We first tried to amplify a ß-lactamase gene from the *N. cyriacigeorgica* clinical strain using primers based on the sequence of *Nocardia cyriacigeorgica* GUH-2 chromosome complete genome GenBank: FO082843.1. The set of primers led to the amplification of a DNA fragment encompassing the ß-lactamase AST-1 precursor GenBank:CCF63029.1. We then analyzed the putative open reading frame resulting from this nucleotide sequence and blast it to assess the sequence conservation. The final nucleotide sequence is displayed in **Figure S1**.

The translation of the nucleotide indicates an open reading frame (ORF) of 333 amino acids **(Table I)**. As ABL are synthesized as a precursor in the cytoplasm and then secreted into the periplasm (Kaderabkova et al., 2022), we explored the possibility of the presence of a peptide signal. We analyzed the sequence with the Signal P server (Teufel et al., 2022) which enabled us to identify a probable secretion signal with a cleavage site between residues Cys54 and Asp55. To further design an optimal sequence for protein expression and crystallization, we made a predictive three-dimensional model with AlphaFold (Jumper et al., 2021) to identify flexible/non-folded regions. This last analysis prompted us to design an ORF ranging from residues Ser71 to Gly333.

Finally, following this design approach, we cloned the nucleotide sequence of Bla_{Ncyr} into an expression vector enabling fusion with the SUMO tag in the N-terminus as well as a multi-histidine tag. We also added between the tag and the coding sequence a sequence encoding for the Tobacco Etch Virus protease to remove the tag during the purification process. This strategy led to the production of a highly soluble form of Bla_{Ncyr}, and following a two-step purification procedure, we could purify about 200 mg of protein from 6 liters of culture with two nickel affinity chromatography steps. The protein was further purified on SEC leading to a very pure and homogeneous protein as **Figure 1(*a*)**.

3.2 Protein assessment quality

To ensure our construct design strategy was successful and led to a well-folded and functional protein, we first assessed the behavior of the protein on SEC. The calibration curve attested that the Bla_{Ncyr} behaves as a homogeneous and stable monomer of an apparent molecular weight (M.W.) of about 29 kDa and as expected from the theoretical M.W. and for this class of enzyme known to be a monomer **(Figure 1(*a*))**. We also conducted a ß-lactamase activity assay. To do so, we used nitrocefin a chromogenic cephalosporin substrate that shifts from yellow to red color upon ß-lactamase activity. The presence of nM concentrations of pure Bla_{Ncyr} triggered a color shift as shown in **Figure 1(*b*)**. The absorbance monitoring assay attested of an increased absorbance at 486 nm in a Bla_{Ncyr} concentration-dependent manner and in contrast to the two control experiments performed without enzyme or with enzyme incubated with avibactam, a covalent inhibitor of β-lactamases. Altogether, these data are in agreement that the construct design was successful and led to the production of a functional protein.

3.3 Crystallization, data collection, and structure determination

We initiated the first crystallization trial at two different protein concentrations 5 and 20.9 $mg.mL^{-1}$. Most drops were clear indicating the very soluble behavior of the protein. Nonetheless, several conditions have promoted the appearance of Bla_{Ncyr} crystals. The best crystals were optimized in sitting drops at a protein concentration of 14 $mg.mL^{-1}$ and using a reservoir solution made of 0.1 M citric acid pH3.5, 10 % PEG 6000, and 6 % ethylene glycol.

A full X-ray dataset was collected and processed to a resolution of 1.25Å **(Table 3)**. The structure was solved by molecular replacement using the prediction model mentioned above as a search request. We finally rebuilt and refined the model to R/Rfree values of 14.5%/17.3% with good geometry **(Table 4)**. The asymmetric unit possesses one monomer. Most of the residues could be rebuilt with the exception of residues 71 to 73 and the G in the N-terminus from the TEV cleavage site. The last G333 in the C-terminus could not be seen as well. In addition, we unambiguously identified two molecules of citrate that were present in the crystallization condition.

3.4 Structural analysis

Bla_{Ncyr} has a classical ABL structure with an α/β hydrolase fold made of two domains **(Figure 2(*a*))**. The first domain is a mixed α/β fold, made of strands β1 to β5 and helices α1 and α9. The second domain is fully α-helical formed by helices 2, 3, 4, 5, 6, 7 and 8 **(Figure 2(*a*))**. The structure is made of nine α-helices and six helices of 3_{10} type. The presence of five β-strands (β1-5) forms one antiparallel β-sheet and completes the structure. For clarity and to match the standard nomenclature of ABL the residue indicated between parentheses are following the Ambler classification (Ambler et al., 1991).

On the contrary to some other class A, we did not notice any disulfide bridge albeit the C121 (C77) and C167 (C123) side chains on helices α2 and α3 are in close proximity. The active site residues and water molecules in proximity are well-ordered and have well-defined electron density map **(Figure 2(*b*))**. The catalytic amino acids are at the interface of the two domains and are composed of residues S114 (S70), K117 (K73), Ser174 (S130), and Glu210 (E166) **(Figure 2(*b*))**. These amino acids are strictly conserved when compared with other ABL structures from other phylae and for which these residues were shown to be crucial for the hydrolysis of β-lactam moiety (Tooke et al., 2019)

Further analysis shows that the class A Bla sequence signature (Philippon et al., 2019) is conserved notably in the three motifs 114-STFK-117 (70-STFK-73), 174-SDN-176 (130-SDN-132), and 278-KTG-280 (234-KTG-236) **(Figure 3)**. The E210 (E166) residue on the so-called Ω-loop, and important for ABL enzymatic activity is also conserved. A detailed classification of β-lactamases based on multiple sequence alignment attested that subclass A1 (Philippon et al., 2019) possesses a clear signature sequence that presents on top of the three motifs mentioned above eighteen additional conserved residues. The Bla_{Ncyr} protein harbors strict conservation of these consensus residues **(Figure 3)** as listed further: R105 (61), R109 (65), T115 (71), G122 (78), A169 (125), N180 (136), D201 (157), R208 (164), N214 (170), D223 (179), T224 (180), T225 (181), T226 (182), P227 (183), W254 (210), R266 (222), W273 (229) and D277 (233).

We searched for the closest structure of Bla_{Ncyr} structure in the entire protein database using the PDBeFold server (https://www.ebi.ac.uk/msd-srv/ssm/ssmstart.html) (Krissinel and Henrick, 2004). Evidently, because of the high number of ABL whose structures were determined, we retrieved numerous similar structures with RMSD below 1 Å. The closest structures were the ABL from *Bacillus licheniformis* (PDB code 1i2s) with a RMSD value of 0.7 Å and 45 % protein sequence identity and more interestingly close similarity (RMSD of 0.78 Å and 46 % protein

sequence identity) with the extended-spectrum β-lactamases CTX-M-15 from *Escherichia coli* (PDB code 4hbt) (Lahiri et al., 2013) **(Figure 2(*c*))**. We found that ABL from species of the same order as *Nocardia*, namely mycobacterium enzymes such as BlaC from *Mycobacterium tuberculosis* (PDB code 2gdn) (Wang et al., 2006) is slightly more distant as it shares a RMSD of 1 Å and 50 % identity. This analysis suggests therefore that Bla_{Ncyr} might have a broad-spectrum substrate specificity as its closest homologues.

Additionally, we could unambiguously identify one citrate molecule that is tightly bound in the vicinity of the active site and as supported by the simulated-annealing Fo-Fc omit map, **(Figure 2(*d*))**. The side chain of Y149 (Y105) interacts through van de Waals interactions **(Figure 2(*e*))**. A network of H-bonds between the side chains of S114 (S70), S174 (S130), T279 (T235), and S281(S237) allow interaction with the citrate molecule, while residues S114 (S70) and N176 (N132) via water molecules mediate specific interactions. The tight binding of citrate is achieved by salt bridges involving K117 (K73), R264 (R220), and K278 (K234). A similar interaction with citrate was previously observed for the structure of the TEM-72, a class A β-lactamase from *Enterobacteriaceae* (Docquier et al., 2011).

Finally, to address if the Bla_{Ncyr} structure is representative of ABL from other *Nocardia* species, we performed multiple sequence alignments of ABL from some of the most frequently encountered human pathogenic *Nocardia* **(Figure 3)**. This sequence analysis demonstrates a close conservation and sequence identity of Bla_{Ncyr} with the *N. nova* 49 %, *N. brasiliensis* 66 %, *N. abscessus* 65 %, *N. pseudobrasiliensis* 65 %, *N. asteroides* 69 %, *N. farcinica* 72 % and *N. otitidiscaviarum* 77 %. All these proteins have almost a strict conservation of the three motifs described earlier and the eighteen residues characterizing class A1. Only a few substitutions were noticed for the class A1 signature in *N. abscessus*, *N. asteroides*, and *N. nova* but probably with no consequence for the enzyme function as they do not concern residues critical for the activity of ABL (Tooke et al., 2019). Thus, this analysis strongly suggests that all ABL from pathogenic *Nocardia* species have very likely similar structures and that all these species may possess functional ABL, and as already shown for *N. farcinica* and *N. asteroides* (Laurent et al., 1999; Poirel et al., 2001).

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Figure legends

Figure 1 Evaluation of the protein quality.

(*a*) Size-exclusion chromatography profile of Bla_{Ncyr} on Superdex 75 Increase 10/300 GL column. The elution profile was compared to protein standard: bovine serum albumin (66 kDa, Ve=9.82 mL), anhydrase carbonic (29 kDa, Ve=12.22 mL), cytochrome C oxidase (12.9 kDa, ve=14.22) and bovine aprotinin (6.5 kDa, Ve=16.33 mL), Ve indicates the elution volume. Bla_{Ncyr} eluted at a volume of 12.18 mL. The void volume (Vo) was determined at 8.53 mL with the volume of elution of thyroglobulin (MW 669kDa). The Bla_{Ncyr} (10 µg loaded) sample used for biochemical and structural analysis is shown on the 15 % Coomassie stained denaturing gel. **(*b*)** Determination of the β-lactamase activity of Bla_{Ncyr} in the presence of nitrocefin. Three concentrations of the enzyme were tested (2 nM, blue curve; 4 nM, green curve; 8 nM, red curve) and two control experiments were performed: one with PBS instead of enzyme and one with avibactam, a potent covalent inhibitor of β-lactamase. The data from three technical replicates were analysed with GraphPad Prism version 10.0.2. On the right of the curves are shown pictures taken at the end of the reaction for each well and attesting to the color shift from yellow to red upon enzymatic reaction.

Figure 2 Structural analysis of Bla_{Ncyr}

(*a*) Cartoon representation of the Bla_{Ncyr} three-dimensional structure in two different orientations. The α/β domain is in blue and the α domain in pink. Nt and Ct stand for N-terminus and C-terminus respectively. **(*b*)** Depiction of the important residues for catalysis of class A β-lactamase activity. The 2Fo-Fc map (blue mesh) contoured at a level of 1σ attests to the high quality of the electron density map. W indicates water molecules that are displayed as a green sphere. Residues in parentheses indicate Ambler numbering. **(*c*)** Structural comparison of Bla_{Ncyr} (blue) with its closest homologues ABL from *Bacillus licheniformis* (Blic, green), *Escherichia coli* (Ec, pink)

and *Mycobacterium tuberculosis* (Mtb, orange). The three catalytic residues shown as sticks are positioned similarly. **(*d*)** Simulated annealing omit map proving the presence of a citrate molecule, shown as a yellow stick and in the proximity of the active site. The map is contoured at a level of 3 σ and represented as a green mesh. **(*e*)** Network of interactions between the citrate molecule and Bla_{Ncyr} residues. The dashed black lines indicate hydrogen bonds. Nitrogen atoms are colored blue and oxygen in red. The interactions were analyzed with the PLIP server (Adasme et al., 2021).

Figure 3 Multiple sequence alignment of class A β-lactamases from human pathogenic *Nocardia*.

The sequence of Bla_{Ncyr} was aligned with the sequences of ABL from *Nocardia otitidiscaviarum* NEB252, *Nocardia farcinica* IFM 10152, *Nocardia brasiliensis* ATCC 700358, *Nocardia asteroides* NCTC11293, *Nocardia nova* SH22a, *Nocardia pseudobrasiliensis* ATCC 51512, *Nocardia abscessus* NBRC 100374. The alignment displays only the sequences matching the Bla_{Ncyr} residues that were modeled in the structure; thus, some of the sequences are missing some residues in the N-terminus. The secondary structure elements from the Bla_{Ncyr} structure are placed above the sequence alignment where α, β, η indicate α-helix, β-sheet and η 3_{10} helix respectively. The figure was generated with the ENDscript server (Robert and Gouet, 2014) https://endscript.ibcp.fr.

Table 1 Macromolecule production information

Source organism: *Nocardia cyriageorgica* clinical strain
DNA source: genomic DNA from *Nocardia cyriageorgica* clinical strain
Forward primer for cloning into the expression vector: 5'-ggtaccgagaacctgtacttccagggttcggccgtggccgatccccggttcgccgcactggaaacg-3'
Reverse primer for cloning into the expression vector: 5'-gtggtgctcgagctaaccgagcacgtcgacgaccgtcctggtcgcgtcggc-3'.
Cloning and expression vector: Champion™ pET SUMO
Complete amino acid sequence of the open reading frame: MRLAAAGARYWPRRSPRWCPSAVTSPASSFRRPGGRRMLAAALALSALAMTAACDSGSTTTA PATSAVTTSAVADPRFAALETTAGARLGVFAVNTGSERTVAHRADERFPMASTFKGLACGALLR EHPLSTGYFDQVIHYSAEELVDYSPVTETRVESGMTVAELCHAAITASDNTAGNQLLKLLGGPQ GFTAFLRSLGDDTSRLDRWETELNTAIPGDERDTTTPAALAADYRALVVGDVLGEPERAQLTA WLVANTTGDTRIRAGLPEDWTVGDKTGSPAYGSALDVAVTWPSGRAPIVIAVLSTKSEQDAEP DNRLVADATRTVVDVLG Complete amino acid sequence of the construct expressed: MGSSHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTT PLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGGGTENLYFQG SAVADPRFAALETTAGARLGVFAVNTGSERTVAHRADERFPMASTFKGLACGALLREHPLSTG YFDQVIHYSAEELVDYSPVTETRVESGMTVAELCHAAITASDNTAGNQLLKLLGGPQGFTAFLR SLGDDTSRLDRWETELNTAIPGDERDTTTPAALAADYRALVVGDVLGEPERAQLTAWLVANTT GDTRIRAGLPEDWTVGDKTGSPAYGSALDVAVTWPSGRAPIVIAVLSTKSEQDAEPDNRLVADA TRTVVDVLG Complete amino acid sequence of the construct after tag removal: GSAVADPRFAALETTAGARLGVFAVNTGSERTVAHRADERFPMASTFKGLACGALLREHPLST GYFDQVIHYSAEELVDYSPVTETRVESGMTVAELCHAAITASDNTAGNQLLKLLGGPQGFTAFL RSLGDDTSRLDRWETELNTAIPGDERDTTTPAALAADYRALVVGDVLGEPERAQLTAWLVANT TGDTRIRAGLPEDWTVGDKTGSPAYGSALDVAVTWPSGRAPIVIAVLSTKSEQDAEPDNRLVAD ATRTVVDVLG

Table 2 Crystallization

Method: vapor drop diffusion in sitting drops
Plate type: Swissci 48-well MRC MAXI Optimization Plates
Temperature: 291 K
Protein concentration: 14 $mg.mL^{-1}$
Buffer composition of protein solution: 20 mM Tris-HCl pH8, 0.2 M NaCl, 0.5 mM β-mercaptoethanol and 5 % glycerol
Composition of reservoir solution: 0.1 M Citric acid, 10% PEG 6000 and 6% ethylene glycol
Volume of the drop: 1 □L+ 1 □L
Reservoir solution: 200 □l

Table 3. Data collection statistics.

	Bla_{Ncyr}
Beamline	PXI-X06SA SLS
Wavelength	0.9999
Resolution range	43.95 – 1.25 (1.28 – 1.25)
Space group	$P\ 3_2\ 2\ 1$
Unit cell (Å,º)	a=b=54.18, c=131.86, α=β=90, γ=120
Total reflections	542218 (27178)
Multiplicity	8.6 (8.8)
Completeness (%)	100 (100)
Mean I/sigma(I)	12 (1)
Wilson B-factor (Å^2)	17.7
R-meas (%)	7.8 (238.6)
CC1/2	0.99 (0.28)

Statistics for the highest-resolution shell are shown in parentheses.

Table 4. Refinement statistics

PDB accession	8QNN
Reflections used in refinement	62994 (4461)
Reflections used for Rfree	2002 (141)
***R*work (%)**	14.51 (26.28)
***R*free (%)**	17.38 (31.20)
Number of non-hydrogen atoms	2196
macromolecules	1960
ligands	26
solvent	210
Protein residues	259
RMS (bonds, Å)	0.007
RMS (angles, º)	0.95
Ramachandran favored (%)	97.28
Ramachandran allowed (%)	2.72
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.00
Clashscore	1.28
Average B-factor (Å^2)	22.11
macromolecules	20.86
ligands	23.91
solvent	33.60

Statistics for the highest-resolution shell are shown in parentheses

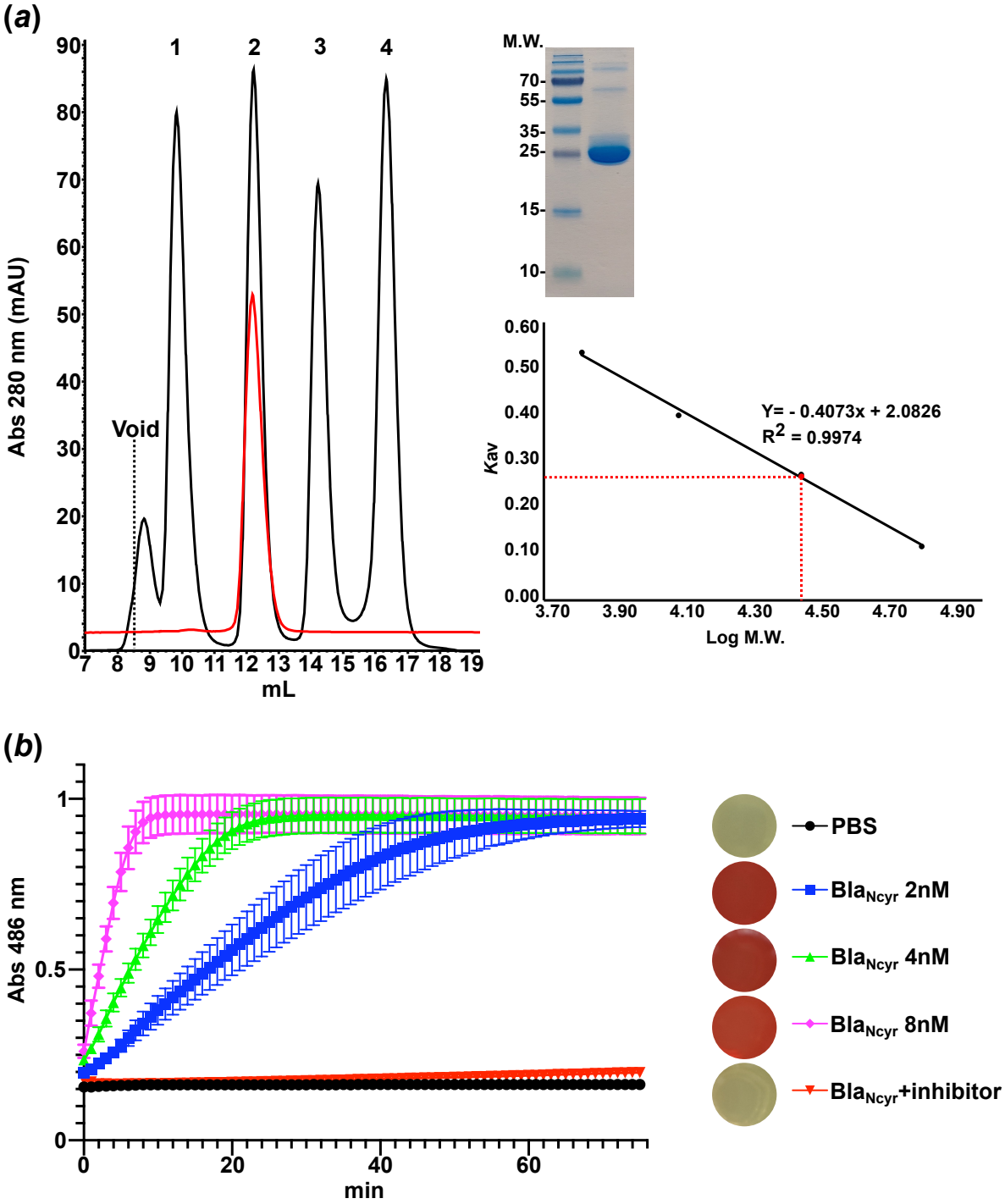
(a)
Abs 280 nm (mAU)
mL
Void
1
2
3
4
M.W.
70-
55-
35-
25-
15-
10-
Kav
Log M.W.
Y= - 0.4073x + 2.0826
R2 = 0.9974
(b)
Abs 486 nm
min
PBS
BlaNcyr 2nM
BlaNcyr 4nM
BlaNcyr 8nM
BlaNcyr+inhibitor

FIGURE 1

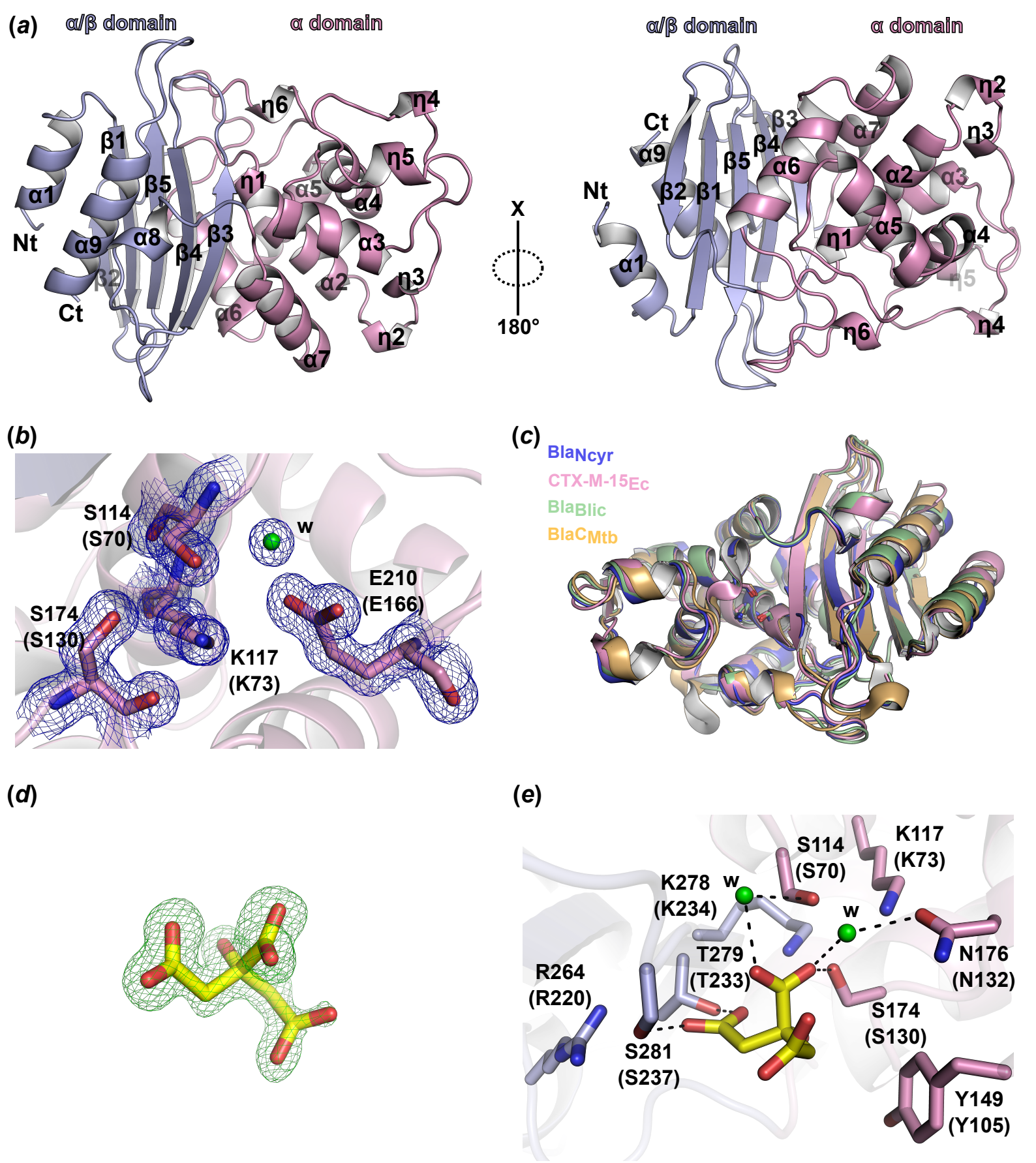
(a)
α/β domain
α domain
α/β domain
α domain
Nt
Ct
β1
β2
β3
β4
β5
α1
α2
α3
α4
α5
α6
α7
α8
α9
η1
η2
η3
η4
η5
η6
X
180°
(b)
S114
(S70)
w
E210
(E166)
S174
(S130)
K117
(K73)
(c)
BlaNcyr
CTX-M-15Ec
BlaBlic
BlaCMtb
(d)
(e)
S114
(S70)
K117
(K73)
K278
(K234)
w
w
T279
(T233)
N176
(N132)
R264
(R220)
S174
(S130)
S281
(S237)
Y149
(Y105)

FIGURE 2

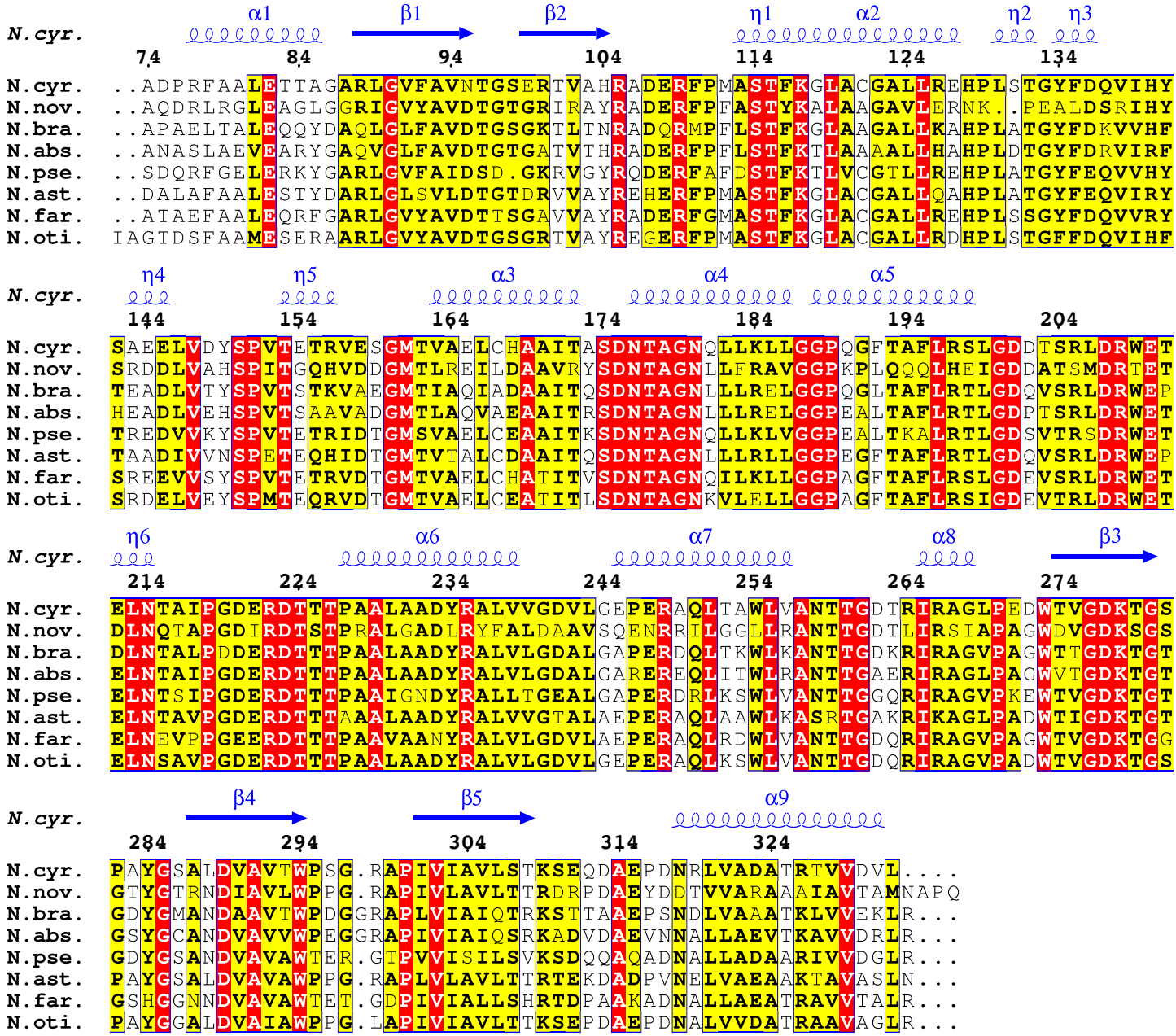
α1 β1 β2 η1 α2 η2 η3
N.cyr. 74 84 94 104 114 124 134
N.cyr. ..ADPRFAALETTAGARLGVFAVNTGSERTVAHRADERFPMASTFKGLACGALLREHPLSTGYFDQVIHY
N.nov. ..AQDRLRGLEAGLGGRIGVYAVDTGTGRIRAYRADERFPFASTYKALAAGAVLERNK..PEALDSRIHY
N.bra. ..APAELTALEQQYDAQLGLFAVDTGSGKTLTNRADQRMPFLSTFKGLAAGALLKAHPLATGYFDKVVHF
N.abs. ..ANASLAEVEARYGAQVGLFAVDTGTGATVTHRADERFPFLSTFKTLAAAALLHAHPLDTGYFDRVIRF
N.pse. ..SDQRFGELERKYGARLGVFAIDSD.GKRVGYRQDERFAFDSTFKTLVCGTLLREHPLATGYFEQVVHY
N.ast. ..DALAFAALESTYDARLGLSVLDTGTDRVVAYREHERFPMASTFKGLACGALLQAHPLATGYFEQVIRY
N.far. ..ATAEFAALEQRFGARLGVYAVDTTSGAVVAYRADERFGMASTFKGLACGALLREHPLSSGYFDQVVRY
N.oti. IAGTDSFAAMESERAARLGVYAVDTGSGRTVAYREGERFPMASTFKGLACGALLRDHPLSTGFFDQVIHF
η4 η5 α3 α4 α5
N.cyr. 144 154 164 174 184 194 204
N.cyr. SAEELVDYSPVTETRVESGMTVAELCHAAITASDNTAGNQLLKLLGGPQGFTAFLRSLGDDTSRLDRWET
N.nov. SRDDLVAHSPITGQHVDDGMTLREILDAAVRYSDNTAGNLLFRAVGGPKPLQQQLHEIGDDATSMDRTET
N.bra. TEADLVTYSPVTSTKVAEGMTIAQIADAAITQSDNTAGNLLLRELGGPQGLTAFLRTLGDQVSRLDRWEP
N.abs. HEADLVEHSPVTSAAVADGMTLAQVAEAAITRSDNTAGNLLLRELGGPEALTAFLRTLGDPTSRLDRWET
N.pse. TREDVVKYSPVTETRIDTGMSVAELCEAAITKSDNTAGNQLLKLVGGPEALTKALRTLGDSVTRSDRWET
N.ast. TAADIVVNSPETEQHIDTGMTVTALCDAAITQSDNTAGNLLLRLLGGPEGFTAFLRTLGDQVSRLDRWEP
N.far. SREEVVSYSPVTETRVDTGMTVAELCHATITVSDNTAGNQILKLLGGPAGFTAFLRSLGDEVSRLDRWET
N.oti. SRDELVEYSPMTEQRVDTGMTVAELCEATITLSDNTAGNKVLELLGGPAGFTAFLRSIGDEVTRLDRWET
η6 α6 α7 α8 β3
N.cyr. 214 224 234 244 254 264 274
N.cyr. ELNTAIPGDERDTTTPAALAADYRALVVGDVLGEPERAQLTAWLVANTTGDTRIRAGLPEDWTVGDKTGS
N.nov. DLNQTAPGDIRDTSTPRALGADLRYFALDAAVSQENRRILGGLLRANTTGDTLIRSIAPAGWDVGDKSGS
N.bra. DLNTALPDDERDTTTPAALAADYRALVLGDALGAPERDQLTKWLKANTTGDKRIRAGVPAGWTTGDKTGT
N.abs. ELNTAIPGDERDTTTPAALAADYRALVLGDALGAREREQLITWLRANTTGAERIRAGLPAGWVTGDKTGT
N.pse. ELNTSIPGDERDTTTPAAIGNDYRALLTGEALGAPERDRLKSWLVANTTGGQRIRAGVPKEWTVGDKTGT
N.ast. ELNTAVPGDERDTTTAAALAADYRALVVGTALAEPERAQLAAWLKASRTGAKRIKAGLPADWTIGDKTGT
N.far. ELNEVPPGEERDTTTPAAVAANYRALVLGDVLAEPERAQLRDWLVANTTGDQRIRAGVPAGWTVGDKTGG
N.oti. ELNSAVPGDERDTTTPAALAADYRALVLGDVLGEPERAQLKSWLVANTTGDQRIRAGVPADWTVGDKTGS
β4 β5 α9
N.cyr. 284 294 304 314 324
N.cyr. PAYGSALDVAVTWPSG.RAPIVIAVLSTKSEQDAEPDNRLVADATRTVVDVL....
N.nov. GTYGTRNDIAVLWPPG.RAPIVLAVLTTRDRPDAEYDDTVVARAAAIAVTAMNAPQ
N.bra. GDYGMANDAAVTWPDGGRAPLVIAIQTRKSTTAAEPSNDLVAAATKLVVEKLR...
N.abs. GSYGCANDVAVVWPEGGRAPIVIAIQSRKADVDAEVNNALLAEVTKAVVDRLR...
N.pse. GDYGSANDVAVAWTER.GTPVVISILSVKSDQQAQADNALLADAARIVVDGLR...
N.ast. PAYGSALDVAVAWPPG.RAPLVLAVLTTRTEKDADPVNELVAEAAKTAVASLN...
N.far. GSHGGNNDVAVAWTET.GDPIVIALLSHRTDPAAKADNALLAEATRAVVTALR...
N.oti. PAYGGALDVAIAWPPG.LAPIVIAVLTTKSEPDAEPDNALVVDATRAAVAGLR...

FIGURE 3

Figure S1: Full nucleotide sequence of Bla_{Ncyr}

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>BlaNcyr

ATGCGACTCGCGGCCGCCGGTGCCCGCTATTGGCCGCGACGATCGCCGCGGTGGTGTCCTTCAGCGGTGACTTCCCC
AGCTTCCTCCTTCCGCCGCCCCGGCGGCAGACGCATGCTCGCAGCCGCACTGGCACTGTCCGCACTGGCGATGACCG
CGGCCTGCGACTCCGGTTCCACCACCACCGCACCGGCCACATCCGCGGTCACCACCTCGGCCGTGGCCGATCCCCGG
TTCGCCGCACTGGAAACGACCGCAGGGGCCCGATTGGGTGTGTTCGCCGTCAACACCGGCTCCGAGCGCACCGTGGC
CCACCGCGCGGACGAGCGGTTCCCGATGGCGTCGACGTTCAAGGGGCTGGCCTGCGGCGCGCTACTACGTGAGCATC
CATTGTCGACGGGCTACTTCGATCAGGTGATCCATTACTCCGCCGAGGAACTGGTCGACTATTCGCCGGTGACCGAA
ACCCGGGTCGAATCCGGGATGACCGTCGCCGAACTGTGCCACGCCGCCATCACGGCCTCCGACAACACGGCCGGCAA
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TGGATCGCTGGGAGACCGAACTGAACACCGCGATCCCCGGCGACGAACGCGACACCACCACCCCCGCCGCACTCGCC
GCCGACTACCGCGCGCTGGTCGTCGGCGACGTACTCGGCGAACCCGAACGCGCCCAGCTGACGGCGTGGCTGGTCGC
CAACACCACCGGCGACACCCGGATCCGCGCGGGTCTGCCCGAGGACTGGACCGTGGGAGACAAGACCGGATCCCCCG
CCTACGGGTCGGCGCTCGACGTCGCCGTCACCTGGCCGTCCGGGCGCGCGCCGATCGTGATCGCGGTGCTGTCGACG
AAGTCCGAGCAGGACGCCGAACCCGACAACAGGCTGGTCGCCGACGCGACCAGGACGGTCGTCGACGTGCTCGGTTA
G
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