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Pilus backbone protein PitB of *Streptococcus pneumoniae* contains stabilizing intramolecular isopeptide bonds

Dorothea Zähner[1,4,*], **Ashish R. Gandhi**[1], **Olga Stuchlik**[3], **Matthew Reed**[3], **Jan Pohl**[3], and **David S. Stephens**[1,2,4]

[1]Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Rollins Research Center, 1639 Pierce Dr., Suite 2101, Atlanta, GA, 30322. USA

[2]Department of Microbiology and Immunology, Emory University School of Medicine, Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322, USA

[3]Biotechnology Core Facility Branch, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30329, USA.

[4]Department of Veterans Affairs Medical Center (Atlanta), 1670 Clairmont Rd., Decatur, GA 30033, USA.

Abstract

Streptococcus pneumoniae type 2 pili are recently identified fimbrial structures extending from the bacterial surface and formed by polymers of the structural protein PitB. Intramolecular isopeptide bonds are a characteristic of the related pilus backbone protein Spy0128 of group A streptococci. Based on the identification of conserved residues in PitB, we predicted two intramolecular isopeptide bonds in PitB. Using a combination of tandem mass spectrometry and Edman sequencing, we show that these bonds were formed between Lys_{63}-Asn_{214} and Lys_{243}-Asn_{372} in PitB. Mutant proteins lacking the intramolecular isopeptide bonds retained the proteolytic stability observed with the wild type protein. However, absence of these bonds substantially decreased the melting temperature of the PitB-derivatives, indicating a stabilizing function of these bonds in PitB of the pneumococcal type 2 pilus.

Keywords

fimbrial protein; intramolecular cross-link; proteolytic stability; thermal stability; mass spectrometry

Introduction

The human pathogen *Streptococcus pneumoniae* (pneumococcus) is a common cause of infections including otitis media, pneumonia, meningitis, and bacteremia [1]. As is common to many pathogenic bacteria, *S. pneumoniae* produces pili, fimbrious extensions on the surface of the bacteria, which can be used for interaction with host tissue and biofilm

*Corresponding Author: VA Medical Center Atlanta, Research Service 151, 1670 Clairmont Rd., Decatur, GA 30033, USA. Phone: +1 404 321-6111, ext. 3854, Fax: +1 404 329-2210, dzahner@emory.edu.

formation [2,3]. Thus, pili are recognized virulence factors, and due to their exposure on the bacterial surface they are regarded as targets for vaccine development.

Two unrelated pilus gene clusters have been identified in *S. pneumoniae* strains, encoding two antigenically different pili: pilus islet 1 (PI-1) [2], and pilus islet 2 (PI-2) [3]. Both pili have the typical features of Gram-positive pili that are composed of covalently polymerized subunits of a pilus backbone protein and may have one or two additional (minor pilus) proteins attached to the backbone structure [4]. The pilus proteins have the typical features of Gram-positive surface proteins that include an N-terminal signal sequence and a C-terminal cell wall sorting signal (CWSS) that contains a LPXTG motif or a variant thereof that was first found in proteins subsequently anchored to the peptidoglycan of the cell wall [5]. The covalent-linkage of pilus subunits, formed by an amide bond between the side chain of a lysine residue and the threonine in the CWSS, requires a pilus-specific sortase [6,7]. The *S. pneumoniae* PI-1 pili are composed of the RrgB pilus backbone protein and the two accessory pilus proteins RrgA and RrgC [2], whereas PI-2 pili are formed solely by the pilus backbone protein PitB [3]. The absence of accessory pilus proteins is a unique feature for PI-2 pili among the so far described pili in Grampositive bacteria, as is the presence of a single PI-2 pilus structure per bacterial cell [3].

In 2007, Kang *et al*. [6] identified intramolecular isopeptide bonds (IPBs) in the pilus backbone protein Spy0128 of the group A streptococcal (GAS) M1 pilus. These IPBs form between the ε-amino group in a lysine residue and an asparagine residue, facilitated by a glutamate [6]. The formation of these IPBs occurs spontaneously [8]. Meanwhile, IPBs have been found in several other pilus backbone proteins in *Bacillus cereus* [9], *Corynebacterium diphtheriae* [10], RrgB of *S. pneumoniae* [11], GBS80 of *Streptococcus agalactiae* [12], and the minor pilus proteins RrgA and RrgC of *S. pneumoniae* [11,13] and GBS52 of *S. agalactiae* [14].

Structural studies of pilus proteins revealed that IPBs are commonly present in protein domains with an IgG-like fold, which has first been described for repetitive CNA B and CNA A domains in the *Staphylococcus aureus* collagen-binding surface protein Cna [15], and that is conserved in many Gram-positive pilus proteins [6,16]. In CNA A domains, as in a CNA B domain in RrgA[13], the formation of the IPB is facilitated by an aspartic acid residue instead of a glutamate residue. However, other surface proteins that are not associated with pili, like the CNA A-similar ACE protein from *Enterococcus faecalis* [17] and the SspB protein from *Streptococcus gordonii* [18] contain IPBs, suggesting a ubiquitous role for this fold and IPBs in Gram-positive surface proteins.

Studies on the function of IPBs in pilus proteins showed that they conferred proteolytic, in particular trypsin stability to pilus proteins [9,11,19]. More recent studies identified an increased thermal stability and mechanical resilience of IPB-containing pilus proteins [19,20] and results obtained with IPB-mutant derivatives in BcpA of *B. cereus* and RrgB of *S. pneumoniae* suggested that IPBs may play a role in pilus biogenesis [9,11].

This communication describes the identification of two IPBs, between Lys_{63} and Asn_{214}, and between Lys_{243} and Asn_{372}, in the PI-2 pilus backbone protein PitB by a combination of tandem mass spectrometry and Edman sequencing. We show that in contrast to IPBs in other pilus protein, the IPBs in PitB were dispensable for proteolytic stability of the pilus protein. However, both IPBs contributed significantly to the thermal stability of PitB, suggesting that they play a stabilizing role for PitB and thereby confer resistance of PitB to physical environmental stresses.

Material and Methods

Construction and purification of IPB deficient derivatives of PitB

The IPB deletion proteins were generated by the exchange of Lys_{63} and Lys_{243}, respectively, to Ala in plasmid-encoded *pitB* [21] by site-specific mutagenesis using the QuikChange II XL site-specific mutagenesis Kit (Stratagene) and the proteins were expressed and purified as previously described for PitB [21]. In addition, the N-terminal 6-His-thioredoxin-tag was removed after cleavage with recombinant enterokinase (Novagen) from the wild type and mutant proteins using His-Pur Cobald-Spin columns (Pierce).

Mass spectrometry and Edman degradation of crosslinked PitB peptides

Recombinant PitB protein was trypsin-digested and chromatographed by reversed-phase high pressure liquid chromatography (RP-HPLC). The masses of peptide fragments were determined by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/ MS), and parent ions were further analyzed by tandem mass spectrometry (MS/MS). N-terminal sequences of the recombinant protein and tryptic fragments were determined by Edman degradation (for experimental details see Supplemental Materials).

Exact mass determination of recombinant PitB and PitB-derivatives

Protein samples were analyzed on a linear trap quadrupole Fourier transform mass spectrometer (LTQ-FTMS, Thermo Scientific). Electrospray ionization mass spectrometry (ESI-MS) spectra were acquired in the ion trap and deconvoluted using ProMass (Novatia).

Proteolytic digestion of PitB and $PitB_{K63A\ K243A}$ and SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

3 μg recombinant PitB and $PitB_{K63A\ K243A}$, respectively, were incubated either with trypsin (Promega; trypsin to protein ratio 1:100 w/w) or added to a culture (at OD_{600nm} = 0.6) of *S. pneumoniae* strain GA47901[21] for 16 hours at room temperature, prior to separation by SDS PAGE (12% Ready gel, BioRad) and staining with Coomassie blue. Heat-denatured protein samples were prepared by boiling (5 min) prior to proteolytic digestion.

Thermal Shift Assays

Thermal shift assays were performed basically as described by El Mortaji *et al*. [11]. In brief, 1 μg of protein and 2 μl of a 1 to 100 dilution of 5000×Sypro Orange (Invitrogen) were added to 50 mM Tris-HCl; 1mM $CaCl_2$ (pH 7.8) buffer to a final volume of 25 μl. Thermal unfolding of the proteins was monitored between 20 °C and 95 °C in 1 °C increments per minutes in a iCycler IQ Real Time PCR machine (BioRad). The melting points were obtained by plotting the negative first derivative of fluorescence against temperature and the minima were referred to as the melting points.

Results

PitB contains the lysine-asparagine-glutamate triads involved in intramolecular peptide bond formation in Spy0128 of S. pyogenes

The pilus islet PI-2 of *S. pneumoniae* shows striking similarity to several pilus gene clusters of group A streptococci [22] in terms of the gene order and the presence of SipA, a signal-peptidase like protein, that was essential for pilus biosynthesis of these two pili, but that was absent from most other Gram-positive pilus islets [3,23]. The crystal structure of the GAS M1 pilus backbone protein Spy0128 was solved and revealed two domains, an N-terminal and a C-terminal CNA B domain that both contained an IPB [6]. However, the sequence similarity between the Spy0128 and PitB protein was very limited, with only 27% identity

between the two proteins. Nevertheless, the alignment between Spy0128 and PitB revealed that the Lys-Asp-Glu residues involved in the IPBs in Spy0128 were all conserved in PitB, i.e., K_{63}-N_{214}-E_{164} and K_{243}-N_{372}-E_{325}, respectively. Also conserved was the described hydrophobic and aromatic amino acid rich environment surrounding the Glu residues that provide the carbanion in the IPB formation in Spy0128 [6]. In addition, PitB encoded a Lys-residue (K195) in the position corresponding to the Lys_{161} in Spy0128 that has been shown to form the isopeptide bond between Spy0128 subunits [6]. Both proteins lack the YPKN-sequence of the described pilin motif that contains the Lys-residue involved in the intermolecular bond formation in most other Gram-positive pilus proteins [24]. In addition, both proteins have non-canonical LP(X)TG-motifs, with VTPTG and EVPTG, respectively, in their C-terminal CWSS (Fig. 1), as has been described earlier [3,25].

PitB contains two intramolecular isopeptide bonds

To determine whether the putative Lys-residues in PitB indeed were involved in IPBs we analyzed the recombinant PitB protein [21] by mass spectrometry. The protein was trypsin digested and the fragments were analyzed for the two predicted non-consecutive peptides that contained IPB1 (Mr 4366.19 Da) and IPB2 (Mr 1216.74 Da), respectively. As shown in Fig. 2, evidence was obtained for the presence of both cross-links in the digests, consistent with the two predicted IPBs K_{63}-N_{214} (IPB1) and K243-N372 (IPB2). The identity of the parent ions with respect to the template sequence of the IPB peptides was confirmed as follows. First, as shown for the parent ion of 1092.56^{+4} m/z (calculated Mr of 4366.19), its mass spectrometry fragmentation pattern was consistent with the predicted cross-linked peptide IPB1 (Fig. 2A). Similarly, fragmentation of a parent ion of 609.38^{+2} m/z (calculated Mr of 1216.75) afforded fragments that could be assigned to their sequence in agreement with cross-linked peptide IPB2 (Fig. 2B). Second, the two tryptic cross-linked fragments originally observed by LC-MS/MS and MALDI-TOF MS in the digests were isolated by off-line capillary reversed-phase high pressure liquid chromatography (RP-HPLC) as distinct entities (Fig. S1 in Supplemental Material), which yielded upon Edman degradation the expected PTH-amino acids in each cycle corresponding to the two peptide chains forming the respective heterodimers (see Supplemental Material for details).

To determine whether PitB may contain additional IPBs or other modifications we analyzed the exact molecular mass of purified recombinant PitB, two single mutants, which lacked one of the IPBs ($PitB_{K63A}$ or $PitB_{K243A}$), and an IPB double mutant ($PitB_{K63A\ K243A}$) (Fig. 2C and Fig. S2 in Supplemental Material). The determined Mr of 37,128.9 Da for the wild type PitB was 34.2 Da lower than the calculated Mr of the linear PitB protein (Fig. 2C). This was consistent with the presence of two IPBs, due to the elimination of two ammonia-groups (17 Da) during the formation of the IPBs. The Mr of both single mutants were 18.0 Da and 15.1 Da lower respectively than calculated for the linear proteins, as expected for the loss of one ammonia group during IPB formation. The Mr of the double mutant $PitB_{K63A\ K243A}$ deviated 1.6 Da from the calculated Mr for the linear peptide, indicating that no other IPBs in an alternative position were formed. Taken together these results demonstrated that only two IPBs were present in PitB and confirmed that these involved Lys_{63} and Lys_{243}, the positions homologous to the IPBs in the pilus backbone protein Spy0128 of *S. pyogenes* M1.

PitB intramolecular isopeptide bonds are not required for proteolytic stability

The first described function of IPBs in a pilus backbone proteins was to confer proteolytic stability [6]. To determine whether the identified IPBs in PitB were conferring trypsin resistance to PitB, the wild type PitB and the IPB double mutant derivative were treated with excess amounts of trypsin and subsequently analyzed by SDS-PAGE (Fig. 3A). Incubation with trypsin did not result in substantial proteolytic digestion of wild type PitB. A characteristic degradation product of about 24 kDa was observed, consistent with a trypsin

susceptibility located between the predicted N-terminal and the C-terminal domains. Surprisingly, the mutant protein appeared to be as trypsin resistant as the wild type protein. The mutant protein produced a characteristic degradation product of 25 kDa, however the overall amount of protein and the amount of degradation product did not differ between the wild type and the mutant PitB, indicating that both proteins were trypsin resistant. Applying longer incubation times did not increase the amount of the degradation products in the wild type sample nor the mutant protein (data not shown). In a control experiment, PitB wild type and mutant protein was heat-denatured prior to trypsin digestion. This treatment resulted in an almost complete degradation of the proteins, with only one band of defined degradation product present, confirming the activity of trypsin under the assay conditions. These results indicated that IPBs were dispensable for trypsin resistance of PitB. Maintenance of trypsin resistance in the absence of IPBs has been observed previously in several IPB single mutants of the PI-1 pilus proteins RrgB (2 out of 3 IPBs) and RrgC (1 out of 2 IPBs) of *S. pneumoniae* [11].

Since PitB forms pilus structures on the surface of *S. pneumoniae*, we asked whether they contribute to proteolytic stability of PitB against pneumococcal proteases, which might be present on the pneumococcal surface or secreted into the medium. PitB and the IPB-double mutant were added to exponentially growing cultures (OD_{600nm}= 0.6) of *S. pneumoniae* strain GA47901 [21]. Stability of PitB and the IPB double mutant appeared not to be affected in the presence of actively growing pneumococci (Fig. 3B). Heat-denatured protein samples of the wild type and the mutant proteins showed no degradation either, suggesting that no pneumococcal proteases with broad substrate specificity were active under our experimental conditions. Thus, our results demonstrate that IPBs were dispensable under natural environmental conditions.

The IPB double mutant (Fig. 3A and B) and the single mutants (data not shown) migrated slightly slower on an SDS-PAGE gel than the wild type protein, with the effect more pronounced for the double mutant. This suggested that the IPBs support a more compact protein conformation. Similar changes in the migration of IPB-containing pilus proteins on SDS-PAGE gels have been observed previously [11,26].

Taken together, we were unable to find evidence for a stabilizing function of IPBs in PitB against proteolysis. Both the wild type and the mutant protein were markedly stable towards trypsin and in their natural environment. It cannot be ruled out that IPBs may protect PitB against other environmentally encountered proteases not present under our experimental conditions, like proteases of bacteria in the same environmental niche or of the host.

Intramolecular peptide bonds confer thermal stability to PitB

To determine whether the IPBs in PitB may contribute to resistance against physical stresses, we analyzed the thermal stability of the wild type protein and the single and double IPB mutant proteins by thermal shift assays [11]. The melting temperature of the wild type protein of 75.9 °C was far higher than for the two single mutants $PitB_{K63A}$ (67.6 °C) and $PitB_{K243A}$ (67.5 °C), respectively (Fig. 4). However, loss of both IPBs in the double mutant $PitB_{K63A\ K243A}$ further decreased the melting temperature to 54.0 °C, demonstrating that the IPBs conferred substantial thermal stability to PitB. Increased thermal stability due to IPBs was observed previously in Spy0128 of *S. pyogenes* and RrgB and RrgC of *S. pneumoniae*, and has been discussed as an indication for a stabilizing function of IPBs for the IgG-like fold [10,11].

Discussion

PitB is similar to M1 pili protein Spy0128 of GAS, with high sequence similarity limited to the conserved residues involved in intra- and intermolecular bonds and their immediate environment (Fig. 1). However, PitB and several GAS pilus backbone proteins [25,27] share additional features that separate these pilus proteins from other Gram-positive pilus proteins, including a modified CWSS with (Q/E/V)(V/T)PTG instead of an LPXTG-motif. In addition, motifs that have been described tentatively to be involved in the formation of IPBs are absent from PitB and these GAS pilus protein: The KVD-like motif [11], which includes the IPB-involved Lys-residue and the E-box motif EXAPXGY [28]. The latter contains a Glu residue, which facilitates IPB formation in *C. diphtheriae* [11]. A KVD-like motif is present in PitB adjacent to K243, with K244 as the lysine of the putative KVD-like motif, but determination of the molecular mass of the mature proteins showed that in the absence of K243 in the $PitB_{K243A}$ mutant K244 is not used alternatively for IPB formation. Therefore, motifs involved in the IPB and intermolecular bond formation in PI-2 and GAS pili still await identification.

The identification of IPBs in PitB that are formed by amino acids residues homologous to those in IPBs of Spy0128 suggests a folding of PitB in an N-terminal and C-terminal domain similar to the two domains observed in the Spy0128 crystal structure [6]. The location of the IPBs delineates the potentially surface exposed regions of PitB. Since in PI-2 pili the backbone protein PitB mediates attachment to eukaryotic cells, in contrast to other Gram-positive pili that employ accessory pilin proteins for adhesion, these exposed regions in PitB are candidates for host interactions and therefore demand further characterization.

In addition to proteolytic and thermal stability, mechanical properties of pilus proteins have been studied. Alegre-Cebollada et al. analyzed the mechanical properties of Spy0128 of GAS and found that this pilus backbone protein was not expandable due to the two IPBs. Supposedly, this rigidity allows M1 pili to withstand forces acting on bacterial surface structures that could otherwise result in the unfolding of the protein [20].

Recently, additional protein modifications in Gram-positive pilus proteins have been described. A disulfide bond was identified in pilus protein SpaA of *C. diphtheriae* [10] and a very unusual intramolecular thioester bond was observed in the adhesin Spy0125 of the GAS M1 pilus, which is formed between a cysteine and a glutamine residue, and is supposedly important in host interaction [29]. However, PitB lacks cysteine residues, ruling out the presence of disulfide or thioester bonds and the determination of the molecular weight of the full length protein is inconsistent with additional protein modifications.

Taken together, we found that PitB has two IPBs, and that these bonds confer thermal stability to PitB, likely stabilizing the overall conformation of the protein. Future work will have to determine the role of these IPBs in the overall pilus structure, whether they are a feature of PI-2 relevant for host-interactions of this important human pathogen and whether they may be sites of conserved epitopes that may be a basis of broadly cross-reactive vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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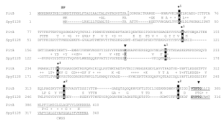

Fig. 1. Alignment of pilus backbone proteins PitB of *S. pneumoniae* and Spy0128 of *S. pyogenes* M1

Residues involved in the formation of isopeptide bonds in Spy0128 are indicated by an asterisk followed by the number for the intramolecular isopeptide bond 1 and 2, and an arrow head for the residues engaged in the intermolecular bond connecting pilus subunits. The region of the mature protein present in the recombinant PitB protein is indicated by square brackets. Signal peptide (SP) and cell wall sorting signal (CWSS) are underlined and the LPXTG-like motif is shown in bold. Accession numbers for PitB and Spy0128 are ADD91687 and AAK33238, respectively. Alignment was performed with ClustalW software.

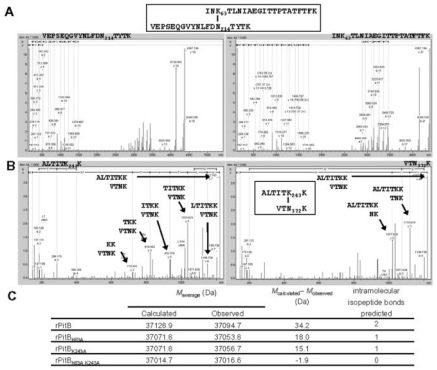

Fig. 2. Identification of intramolecular isopeptide bonds (IPBs) between Lys_{63}-Asn_{214} (IPB1) and Lys_{243}-Asn_{273} (IPB2) of *S. pneumoniae* PitB

MS/MS spectra of IPB1-containing parent ion 1092.6^{4+} m/z (mass to charge ratio) (A) and IPB2-containing parent ion 609.4^{2+} m/z (B). The peptides corresponding to the y-ions are indicated. (C) Exact mass determination of recombinant PitB and IPB-derivatives.

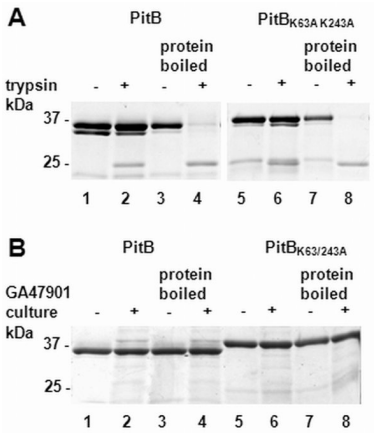

Fig. 3. Proteolytic stability of recombinant PitB and PitB$_{K63A\ K243A}$

Recombinant PitB (lanes 1–4) and PitB$_{K63A\ K243A}$ (lane 5–8) were treated either with trypsin (A) or added to a culture of *S. pneumoniae* strain GA47901 (B) prior to separation by SDS PAGE. Whether trypsin or culture was present in the reaction and whether the samples were boiled prior to incubation is indicated on top of the gels.

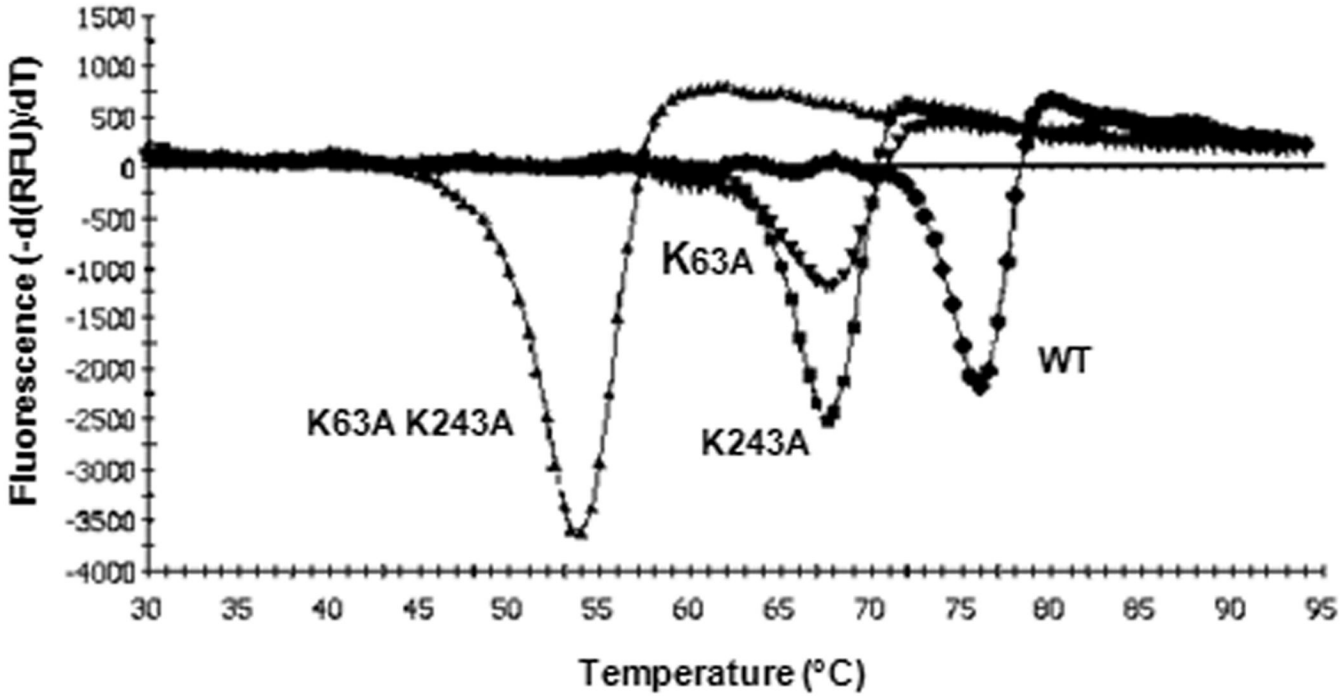

Fig. 4. Thermal shift assays with recombinant PitB and PitB-derivatives

Melting points were determined for PitB (WT, 75.9 °C; ●), $PitB_{K63A}$ (67.6 °C; ▼), $PitB_{K243A}$ (67.5 °C; ■), and $PitB_{K63A\ K243A}$ (54.0 °C; ▲).