Cloning, Overexpression, and Characterization of a Novel Thermostable Penicillin G Acylase from *Achromobacter xylosoxidans*: Probing the Molecular Basis for Its High Thermostability

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The gene encoding a novel penicillin G acylase (PGA), designated *pgaW*, was cloned from *Achromobacter xylosoxidans* and overexpressed in *Escherichia coli*. The *pgaW* gene contains an open reading frame of 2,586 nucleotides. The deduced protein sequence encoded by *pgaW* has about 50% amino acid identity to several well-characterized PGAs, including those of *Providencia rettgeri*, *Kluyvera cryocrescens*, and *Escherichia coli*. Biochemical studies showed that the optimal temperature for this novel PGA (PGA650) activity is greater than 60°C and its half-life of inactivation at 55°C is four times longer than that of another previously reported thermostable PGA from *Alcaligenes faecalis* (R. M. D. Verhaert, A. M. Riemens, J. V. R. Laan, J. V. Duin, and W. J. Quax, Appl. Environ. Microbiol. 63:3412–3418, 1997). To our knowledge, this is the most thermostable PGA ever characterized. To explore the molecular basis of the higher thermostability of PGA650, homology structural modeling and amino acid composition analyses were performed. The results suggested that the increased number of buried ion pair networks, lower N and Q contents, excessive arginine residues, and remarkably high content of proline residues in the structure of PGA650 could contribute to its high thermostability. The unique characteristic of higher thermostability of this novel PGA provides some advantages for its potential application in industry.

Penicillin G acylase (PGA; EC 3.5.1.11) is an important enzyme in the bulk pharmaceutical industry. It has been used to hydrolyze benzylpenicillin to generate phenylacetic acid and 6-aminopenicillanic acid; the latter is a key intermediate in the synthesis of a large variety of semisynthetic penicillins and cephalosporins (36, 37). Previous studies showed that PGA is a heterodimeric protein consisting of a small α subunit and a large β subunit. The PGA enzyme is synthesized as an inactive preprotein that contains a leader peptide directing the protein to its destination and a spacer peptide separating the α and β subunits. Formation of active PGA includes a series of post-translational steps via translocation and periplasmic processing and folding that are unusual for prokaryotic proteins (39). The PGA enzyme belongs to the family of N-terminal nucleophile (Ntn) hydrolases, a class of enzymes that all have a fold around the active site and contain a catalytic nucleophile at the N-terminal position, whereas in PGA, the catalytic nucleophile is a serine that is located at the N-terminal position of the large β subunit (10). Expression of *Escherichia coli* PGA is usually modulated by several factors, such as growth temperature, phenylacetic acid, oxygen levels, strain variations, and glucose in both wild-type and recombinant *E. coli* (20, 27). A normal

growth temperature is favorable for its expression, and it is subject to induction by phenylacetic acid.

Since the rate of an enzymatic reaction generally increases with temperature, the application of an enzyme with greater temperature stability will improve the efficiency of many industrial processes (21). Early studies suggested that the characteristics commonly associated with elevated thermostability in proteins include a relatively small solvent-exposed surface area; an increased packing density, which reduces cavities in the hydrophobic core; optimization of hydrophobic interactions; decreased length of surface loops; and hydrogen bonds between polar residues (49, 50). Approaches comparing the amino acid sequences and crystal structures of homologous mesophilic and thermophilic proteins have been used to determine structural features contributing to the thermostability of many proteins, and such knowledge will be essential for the rational design of highly thermostable protein by protein engineering techniques (35).

During our effort to obtain cephalosporin acylase-producing organisms, a strain capable of producing both a cephalosporin acylase and a PGA was isolated. The strain was later identified as *Achromobacter xylosoxidans* subsp. *xylosoxidans* (synonym, *Alcaligenes xylosoxidans* subsp. *xylosoxidans*) (data not shown). It is interesting that *A. xylosoxidans* has been suggested as an important cause of bacteremia in immunocompromised patients, and the strains involved are usually multiply resistant to antimicrobial therapy. Some recent findings suggest that *A. xylosoxidans* subsp. *xylosoxidans* is also an emerging pathogen in cystic fibrosis (51). In this paper, we describe the cloning and

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characterization of a novel PGA (PGA650) from *A. xylosoxidans*. The gene that encodes it was overexpressed in *E. coli*, and the PGA enzyme was purified and used for kinetic analyses. The results showed that PGA650 possesses the characteristic of higher thermostability, which provides significant advantages over other well-characterized penicillin acylases in β -lactam conversions for its potential application in industry. To further understand the molecular mechanism of its enhanced thermostability, the enzyme structure was modeled on the basis of the determined tertiary structures of *E. coli* and *P. rettgeri* penicillin G amylases, and the possible structural characteristics associated with its thermostability were investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *A. xylosoxidans* strain used in this study is from our laboratory collection. *E. coli* strains DH5 α and JM109 (laboratory stock) were used for routine cloning procedures and propagation and amplification of all plasmid constructs. Overproduction of the PGA was performed with *E. coli* BL21(DE3) (Novagen). The *E. coli* and *A. xylosoxidans* strains were routinely grown and maintained in Luria-Bertani medium with appropriate antibiotic selection when needed. Bacterial cultures for enzyme expression assays were grown in Luria-Bertani broth left unsupplemented or supplemented with phenylacetic acid. Cultures were incubated at 22 or 28°C for 48 h or at 37°C for 24 h.

Gene cloning and sequencing. Chromosomal DNA of *A. xylosoxidans* was isolated and partially digested with Sau3A. Fragments of 3 to 5 kb were purified and ligated into the BamHI-opened, dephosphorylated vector pBluescriptIISK(+) and then transformed into *E. coli* DH5 α (*proA leuB thi recA*) on plates containing ampicillin. Recombinant clones harboring the gene for PGA were selected by an overlay technique using *Serratia marcescens* as described by Meevootisom et al. (26). The positive clone (JM109/pPGA) was obtained, and the insertion was sequenced by the dideoxy method.

Protein expression and purification. PGA650-encoding gene *pgaW* was amplified by PCR with the JM109/pPGA plasmid as the template with two oligonucleotides, pa650L (5'-CATATGTAGCGGCTCCGTATCATGGT-3') and pa650R (5'-AAGCTTCCTCGGAGTCGACGGTATC-3'). The resulting 2.9-kb PCR fragment was cloned into the EcoRV site of pBluescriptIISK(+) to generate pKS_{pga}. pKS_{pga} was doubly digested with HindIII and NdeI, and the released insert was cloned into pET28b(+) (Novagen) to generate pPGA_w. pPGA_w was introduced into *E. coli* strain BL21(DE3) (Novagen) to allow expression of *pgaW* under the control of the T7 *lac* promoter. The cells were harvested by centrifugation at 4,000 rpm for 20 min in a Beckman J2-HS instrument and resuspended in 30 ml of osmotic-shock buffer (33 mM Tris-HCl [pH 7.3], 1.5 mM EDTA, 40% sucrose) after induction with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 3 to 4 h at 28°C. A cold osmotic-shock procedure was performed in accordance with reference 15. The final volume of periplasmic extracts was 10 ml. The periplasmic extract fraction precipitated with ammonium sulfate at 30 to 60% saturation was loaded directly onto a hydrophobic interaction phenyl Sepharose CL-4B column (Amersham Pharmacia Biotech). Fractions containing PGA activity were pooled, concentrated by ultrafiltration (cutoff of 30 kDa), and dialyzed in 20 mM Tris-HCl (pH 7.5). The resulting sample was applied to an ion-exchange Mono Q HR5/5 column (Amersham Pharmacia Biotech). The active fractions were pooled and desalted. After they were boiled for 5 min in loading dye, two protein bands of 27.0 and 62.4 kDa were revealed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE). All purification steps were carried out at room temperature in a fast protein liquid chromatography system (Amersham Pharmacia Biotech).

Sequence analysis. The purified protein was analyzed by SDS-PAGE. After electrophoresis, the protein bands were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. Bands corresponding to the α and β subunits were cut out. The amino acid sequence of the N terminus was determined by automated Edman degradation with an ABI 492 Procise cLC Sequenator.

DNA and protein sequences were analyzed by using the Wisconsin Genetics Computer Group sequence analysis package (version 8.1). Amino acid similarity and identity percentages were determined by ClustalW multiple-sequence alignment (43). The World Wide Web Prediction Server SignalP V1.1 (<http://www.cbs.dtu.dk/services/SignalP/>) (32) was used to predict the presence and locations

of signal peptide cleavage sites in amino acid sequences. Evolutionary relationships were analyzed by using the PROTDIST, FITCH, DRAWGRAM, and DRAWTREE routines of the PHYLIP 3.6a program, which is available at the Pasteur Institute server (<http://bioweb.pasteur.fr/seqanal/phylogeny/phytip-uk.html>) (11, 12).

Enzyme assay and characterization. The specific activity of PGA650 and kinetic parameters for hydrolysis were determined as described by Yang et al. (52). The model substrate, 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) and its natural substrate penicillin G were used for the assay. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μ mol of NIPAB per min at 37°C and pH 8.0. The protein concentration was determined by the Bradford method.

The optimal temperature for the activity was determined in a range of 30 to 70°C by measuring NIPAB hydrolysis. The effect of pH on the activity of the enzyme was determined with NIPAB as the substrate at 37°C in 50 mM phosphate buffer (pH 6 to 8) or 50 mM Na₂HPO₄-NaOH (pH 9).

The PGA of *E. coli* D816(pDB3) was used as a control for the thermostability assay (7). Twenty-microliter aliquots of the purified enzyme that had been diluted in 50 mM sodium phosphate buffer (pH 8.0) were incubated for 0, 5, 10, 20, 25, 30, 35, 40, 45, 50, or 55 min at 60 or 55°C, respectively. After cooling of the samples, the activity was determined by using a fixed, saturating NIPAB concentration (0.9 mg/ml) at 37°C.

Structural analysis by comparative-modeling techniques. The three-dimensional structures of the PGAs of *P. rettgeri* (PDB [Protein Data Bank] code 1CP9) and *E. coli* (PDB codes 1AJN and 1E3A) were extracted from the Brookhaven PDB (9, 18, 25). The structures were superimposed, and a sequence alignment was derived from the structural equivalences by the Insight II program (ACCELRYS 1997, San Diego, Calif.). The *A. xylosoxidans* PGA sequence and the above three sequences extracted from the PDB (1CP9, 1AJN, and 1E3A) were then aligned by using the ClustalW program (43) and manually manipulated to optimize the matching of several structural characteristics. Side chain substitutions were automatically done by the Insight II program. A distance-dependent dielectric constant, no Morse potential, no cross-terms, and charges "on" were used for the 100 steepest-descent and 500 conjugate gradient energy minimization steps. All geometry optimization operations were performed with the consistent valence force field in the Discover program from Molecular Simulation Inc. The HOMOLGY package in Insight II (ACCELRYS 1997) was used for manipulation of structures and alignments. PROCHECK (24, 28) was used to monitor the stereochemical quality of the final models, and PROSAII (38) was used to measure overall protein quality in terms of packing and solvent exposure. Secondary structures were determined by using the program DSSP (19), and the salt bridges were calculated through the WHAT IF Web Interface (<http://www.cmbi.kun.nl/gv/servers/WIWWWI/>) (33). A salt bridge is defined as a negative atom (side chain oxygens in Asp or Glu) and a positive atom (side chain nitrogens in Arg, Lys, or His) with an interatomic distance of less than 7.0 Å. All of the programs were run under IRIX 6.3 on O2 silicon Graphics stations.

Nucleotide sequence accession numbers. The nucleotide and the protein sequences of PGA650 reported here have been submitted to the GenBank database under accession no. AF490005.1 and AAP20806, respectively.

RESULTS AND DISCUSSION

Cloning and sequencing of the *pgaW* gene from *A. xylosoxidans*. The *A. xylosoxidans* genomic library was constructed by inserting partially digested chromosomal DNA into the vector pBluescriptIISK(+) and screened by an overlay technique using *S. marcescens*, which is resistant to penicillin G but sensitive to 6-aminopenicillanic acid (26). A positive clone harboring *pgaW* named JM109/pPGA was obtained. The clone carries a 3.5-kb insert and was double strand sequenced. Sequence analysis of the fragment indicated that it contained the complete *pgaW* gene homolog with an open reading frame of 2,586 nucleotides (accession no. AF490005.1). The G+C content of the *pgaW* gene is 68.72%, which is higher than that of most of the PGA-encoding genes reported so far, such as the *Thermobifida fusca* Tfus_32 putative *pga* gene (67.88%) and those of *E. coli* (47.82%), *Alcaligenes faecalis* (56.51%), *Arthrobacter viscosus* (36.23%), and *Bacillus megaterium* (35.69%).

FIG. 1. Phylogenetic tree of determined and putative prokaryotic PGAs based on the enzymes' amino acid sequences. The tree was constructed with the ClustalW and PHYLIP 3.6a programs by the neighbor-joining method as described in Materials and Methods. The scale bar represents 10 amino acids residues substitutions per 100 amino acids residues.

Amino acid and N-terminal sequence analyses of PGA650.

The deduced protein sequence encoded by *pgaW* showed the typical PGA polypeptide organization (signal sequence- α subunit-spacer peptide- β subunit). The first stretch of 21 amino acids has the property of a signal peptide, substantiating the periplasmic location of the enzyme. The α subunit contains 247 amino acid residues, whereas the β subunit contains 557 amino acids starting from the N-terminal serine.

The amino acid sequence of PGA650 was compared to the sequences of other characterized PGAs by standard search algorithms (BLASTP). The results showed that PGA650 has 51% amino acid identity with the PGA (P06875) of *E. coli*, 52% identity with the PGA (A56681) of *P. rettgeri*, 52% identity with the PGA (P07941) of *Kluverella cryocrescens*, 41% identity with the PGA (AAL58441) of *A. faecalis*, 28% identity with the PGA (AAD45609) of *B. megaterium*, and 28% identity with the PGA (P31956) of *A. viscosus*.

A phylogenetic tree of several known PGAs and PGA650

was constructed with the PHYLIP 3.6a program (Fig. 1). It showed that PGA650 from *A. xylosoxidans* is phylogenetically close to the well-characterized PGAs from *Providencia rettgeri* (A56681), *A. faecalis* (AAL58441), *K. cryocrescens* (P07941), *E. coli* (P06875), *B. megaterium* (AAD45609), and *A. viscosus* (P31956). Included in the phylogenetic tree, PGAs from *Ralstonia metallidurans* (ZP_00027232), *Pseudomonas aeruginosa* PA01 (NP_250584), (NP_248996), *Pseudomonas putida* KT2440 (NP_747265), *Pseudomonas fluorescens* PfO-1 (ZP_00085714), and *A. xylosoxidans* (AAP20806) interested us because these strains are all gram-negative nonfermenting bacilli and are opportunistic human pathogens that are major causes of chronic lung infection in patients with cystic fibrosis (14). They also share the characteristics of intrinsic and acquired resistance to multiple antimicrobial agents (34). Multidrug-resistant pathogens are probably a potential source of β -lactam acylase.

N-terminal sequence analysis failed to determine the N-terminal sequence of the small subunit (data not shown). It is

FIG. 2. SDS-PAGE analysis of PGA650 purified from recombinant *E. coli* BL21(DE3)/pPGA650. The molecular weight standard in lane M was stained with 0.025% Coomassie brilliant blue R-250 after electrophoresis. The molecular masses of marker proteins are indicated in kilodaltons. The enzyme (lane E) was purified from *E. coli* BL21(DE3)/pPGA650 as described in Materials and Methods. The bands corresponding to the α and β subunits of PGA650 are indicated on the right.

possible that the supposed N-terminal glutamine (QPVAQA AGQ) residue was blocked by pyroglutamine formation, making the protein inaccessible to automated degradation. A similar blockage was also reported for the α subunit of *A. faecalis* PGA (46). The N-terminal sequence of the β subunit was determined to be SNMWIVGRDHAKDARS, which contains the conserved N-terminal serine-asparagine sequence found in the β subunits of the all known PGAs.

Overexpression and purification of PGA650. The optimal growth temperature for PGA650 expression is 28°C. This study found that higher cultivation temperatures, such as 37°C, led to lower specific activity of PGA650, while lower cultivation temperatures, such as 22 or 25°C, resulted in poor biomass for further protein isolation. Under the optimal condition, a typical 4,000 U of PGA650 activity can be achieved from a 1-liter *E. coli* culture.

The overexpressed PGA650 enzyme was purified from *E. coli* BL21(DE3)/pPGA650 with a degree of purity of about 99%. The purified enzyme showed two bands on SDS-PAGE, corresponding to the α and β subunit with molecular masses of approximately 27.0 and 62.4 kDa, respectively (Fig. 2). The size of the β subunit was similar to that of other known PGAs, while the size of the α subunit was larger than that of other known PGAs.

Characterization and kinetic properties of recombinant PGA650. The expression analysis demonstrated that PGA650 can be synthesized and folded properly in *E. coli*. However, phenylacetic acid, which has been reported to be an expression inducer for PGAs from many other sources, including *E. coli*, has no induction effect on PGA650 synthesis (specific activity) in *E. coli* (data not shown). The biochemical characteristics (molecular mass, enzymatic activity, and activity dependency on temperature and pH) were determined for both purified

FIG. 3. Effects of temperature and pH on the activity of recombinant purified PGA650. (A) Temperature-activity profile of PGA650. The activity of the enzyme was assayed at each temperature in 50 mM phosphate buffer (pH 8) by measuring NIPAB hydrolysis. (B) pH-activity profile of PGA650. The effect of pH on the activity of the enzyme was determined at 37°C in 50 mM phosphate buffer (pH 6 to 8) and 50 mM Na_2HPO_4 -NaOH (pH 9) for the NIPAB substrate.

recombinant enzyme and the native enzyme, which were similar and were comparable to those reported other PGAs. The temperature and pH dependence analysis of PGA650 suggested that the optimal temperature for PGA650 activity is about 60°C (Fig. 3A), while the optimal temperatures for *E. coli* PGAs are normally 30 to 55°C. The optimal pH for hydrolytic activity was 8.5 (Fig. 3B). Activity decreased marginally when the pH was lowered to 5.3.

Kinetic parameters of PGA650 were determined (Table 1). The K_m values for NIPAB and penicillin G were 27.0 ± 1.0 and 8.9 ± 0.4 μM , respectively. The turnover rates (k_{cat}) for penicillin G and NIPAB were 68.8 ± 3.8 and 72.7 ± 3.0 s^{-1} , respectively. Its activity was inhibited strongly by phenylmeth-

TABLE 1. Enzymatic parameters of purified *A. xylosoxidans* PGA

Substrate	Avg K_m (μM) $\pm$ SD	Avg k_{cat} (s^{-1}) $\pm$ SD	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$), 10^6
NIPAB	27.0 ± 1.0	68.8 ± 3.8	2.6
Penicillin G	8.9 ± 0.4	72.7 ± 3.0	8.2

FIG. 4. Thermal inactivation of purified recombinant PGAs from *A. xylosoxidans* and *E. coli*. A 20- μ l sample of the purified enzyme diluted in 50 mM sodium phosphate buffer (pH 8.0) was incubated in Eppendorf tubes at 60 or 55°C. At the indicated times, samples (20 μ l) were withdrawn, cooled, and then tested for enzyme activity at 37°C by using a fixed, saturating NIPAB concentration (0.9 mg/ml). The calculated $t_{1/2}$ s at 60 and 55°C for the recombinant PGA650 enzyme were about 8 and 55 min, respectively. The symbols $\blacklozenge$ and $\blacktriangle$ represent the thermal stability of the *A. xylosoxidans* enzyme at 55 and 60°C, respectively; the symbols $\blacksquare$ and $\times$ represent the thermal stability of PGA from *E. coli* at 55 and 60°C, respectively.

ylsulfonyl fluoride, a specific inhibitor of serine proteinase, and the K_i value is 0.1 μ M.

PGA650 has the greatest thermostability of the PGAs reported. The high optimal temperature for its activity is a striking feature of PGA650. However, the most remarkable property exhibited by this protein is its high intrinsic thermostability. The half-life of inactivation ($t_{1/2}$) was determined for the purified PGA650 protein. The results showed that PGA650 has a $t_{1/2}$ of 55 min at 55°C and 8 min at 60°C, compared to the 15 min at 55°C for PGA from *A. faecalis*, which was perhaps the most thermostable PGA known before (46). The results demonstrated that *A. xylosoxidans* PGA is intrinsically highly thermostable (Fig. 4). To our knowledge, no other characterized PGA is as capable of withstanding heat denaturation as PGA650 is.

Molecular basis for the high thermostability of PGA650: study by homology modeling approach. To obtain further information about the molecular mechanism of the enhanced thermostability of PGA650, a homology modeling approach was applied to compare the PGA650 enzyme and the PGAs of *E. coli* and *P. rettgeri*. A molecular model of *A. xylosoxidans* PGA was constructed by using the known tertiary structures of the PGAs of *E. coli* and *P. rettgeri*, which have high homology with PGA650 (more than 50%). The modeling analysis showed that one of the major structural features of the *A. xylosoxidans* enzyme is a significantly increased number of buried ion pair networks. The WHATIF software atomic contacts-salt bridges calculation results showed that the *A. xylosoxidans* enzyme has approximately 6% more salt bridges than that of *E. coli* and about 30% more salt bridges than that of *P. rettgeri* (Table 2). The networks of ion pairs that are found in the *A. xylosoxidans* enzyme are reliant on neighboring ionic and hydrogen bonding interactions. An early study has suggested that there is a strong

correlation between the number of salt bridges and protein thermal stability (8). Ion pairs are important in stabilizing proteins that operate near the upper temperature limits of protein stability (45). A single salt bridge can contribute 13 to 22 kJ/mol to the free energy of folding, and salt bridges are relatively unaffected at extremely high temperatures. They may therefore provide the large increments of structural stability necessary to maintain stability at or above 100°C (47). It is possible that the presence of extra ion pairs is the primary source of thermostabilization in the *A. xylosoxidans* enzyme.

Molecular basis for the high thermostability of PGA650: analysis of amino acid composition. The amino acid composition of PGA650 and its correlation with thermostability were investigated. The results showed that the PGA650 protein has decreased amounts of amino acid residues of N plus Q and of S plus T (Table 2), which is consistent with the conclusion of the genomic studies conducted by Chakravarty and Varadarajan (5) and Sterner and Liebl (40). They found that mesophilic proteins normally have a decreased content of uncharged polar amino acids (i.e., Q, N, S, and T) and the hyperthermophilic proteins have an increased content of charged amino acid residues (i.e., K, E, and R) (4, 5, 16, 42, 48). As S and T can catalyze the deamination and backbone cleavage of Q and N residues (5, 40), a reduction in the amounts of all four of these residues would minimize deamination. Reduction of deamidation by decreasing the amounts of glutamine and asparagine in proteins confers stability on thermophilic proteins.

There is also a markedly high content of Ala residues in helices in *A. xylosoxidans* PGA650 compared with the PGAs from other sources. These extra Ala residues may contribute to the thermostability of these enzymes, not only by significantly increasing the hydrophobicity of the subunit interaction but also by stabilizing the individual helices since alanine can serve the helix-forming function (2).

The high content of arginine residues in *A. xylosoxidans* PGA and the higher Arg/Lys ratio may also account for its high thermostability to some degree (Table 2). Early studies indicated that there is a correlation between protein stability and the number of arginines on the protein surface (44). Thermophilic proteins have, on average, a higher arginine content and a greater amount of reducing lysines on the protein surface (1, 48). The site-directed mutagenesis study has proved that Lys-Arg mutations increases the thermotolerance of enzymes (6, 29, 44) because of stronger hydrogen bonding of the large guanidinium group of arginine with nearby polar groups (44). Because of its large side chains, arginine may be important in both local and long-range interactions (3, 22). In addition, arginine residues may also provide advantages for thermostable proteins in the following ways: involvement in ion pair networks, better shielding of the hydrophobic hydrocarbon

TABLE 2. Putative important factors responsible for the high thermostability of *A. xylosoxidans* PGA

PGA source	No. of salt bridges	% N + Q	% S + T	% Ala	% Arg	Arg/Lys ratio	% Pro
<i>A. xylosoxidans</i>	506	8.96	9.08	13.68	7.21	13.68	6.84
<i>E. coli</i>	478	11.93	12.71	8.76	4.23	8.76	5.15
<i>P. rettgeri</i>	392	14.02	12.82	6.68	2.67	6.68	5.47

chain from H₂O, more rigidity, and remaining charged at higher pHs.

Another feature that may be related to the thermostability of PGA650 is its notably greater amount of proline residues (Table 2). Proline has a unique geometry, restricting the conformational flexibility of the folded polypeptide chain (13, 23); proline also fits well into the first turn of an α -helix and consequently increases internal hydrophobicity, thus stabilizing the α -helix (41). In addition, it was stated that proline residues, in general, reduce the entropy of unfolding and are therefore important in stabilizing the molecular structure. It has been suggested that introduction of prolines at selected sites in target proteins increases their molecular stability (17, 30, 31, 53).

In this paper, we have reported the cloning and characterization of a novel thermostable PGA from *A. xylosoxidans* and explored the possible molecular mechanism responsible for its thermostability. The results suggested that the increased buried ion pair networks, decreased N and Q content, and high arginine and proline content could contribute to the thermostability of *A. xylosoxidans* PGA650. The PGA650 protein is the most thermostable penicillin acylase reported so far. Its thermostability makes the *A. xylosoxidans* PGA650 enzyme a more attractive biocatalyst both in hydrolysis and in synthetic conversions. A detailed study is under way to determine the structural features that confer this high thermostability.

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