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Enzymes from cold-adapted microorganisms

The class C β-lactamase from the antarctic psychrophile *Psychrobacter immobilis* A5

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A heat-labile β-lactamase has been purified from culture supernatants of *Psychrobacter immobilis* A5 grown at 4°C and the corresponding chromosomal *ampC* gene has been cloned and sequenced. All structural and kinetic properties clearly relate this enzyme to class C β-lactamases. The kinetic parameters of *P. immobilis* β-lactamase for the hydrolysis of some β-lactam antibiotics are in the same range as the values recorded for the highly specialized cephalosporinases from pathogenic mesophilic bacteria. By contrast, the enzyme displays a low apparent optimum temperature of activity and a reduced thermal stability. Structural factors responsible for the latter property were analysed from the three-dimensional structure built by homology modelling. The deletion of proline residues in loops, the low number of arginine-mediated H-bonds and aromatic-aromatic interactions, the lower global hydrophobicity and the improved solvent interactions through additional surface acidic residues appear to be the main determinants of the enzyme flexibility.

Keywords: psychrophile; extremophile; Antarctic; β-lactamase; cephalosporinase.

Psychrophilic microorganisms represent a major class of the microbial world if one considers the vast extent of permanently cold environments on Earth (deep-sea waters, polar and alpine regions). In spite of the diversity and abundance of these extremophiles, the numerous physiological and biochemical adaptations to the life at temperatures close to 0°C remain poorly documented [1, 2]. The dominating character of cold-adapted enzymes is probably their enhanced turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m): improving these kinetic parameters compensates for the reduction of reaction rates at low temperatures and adequate metabolic fluxes are therefore maintained [3]. According to the current hypothesis [4], optimization of the catalytic parameters can originate from the highly flexible structure of these proteins which provides enhanced abilities to undergo conformational changes during catalysis at low temperatures. The usually observed thermal lability is therefore regarded as a consequence of the folded structure flexibility. The molecular origin of this flexibility has been analysed from the primary structure of some psychrophilic bacterial enzymes. All studies suggest a potentially low number of weak interactions stabilizing the folded conformation [5, 6]. We report here the kinetic characterization, the nucleotide sequence and a structural analysis of the β-lactamase secreted by the antarctic psychrophile *Psychrobacter immobilis* A5, a gram-negative bacterial strain collected in an environment ranging in temperature from −20 to +2°C. A brief account of some properties of another β-lactamase from a closely related strain has been given recently [7].

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Enzyme. β-lactamase (EC 3.5.2.6).

Note. The novel nucleotide sequence data reported here have been submitted to the GenBank/EMBL sequence data banks and are available under the accession number X83586.

Bacterial β-lactamases hydrolyse the amide bond of the β-lactam ring of penicillin-derived antibiotics yielding biologically inactive compounds. Due to their critical role in bacterial antibiotic resistance, the substrate specificities and the action mechanisms of mesophilic β-lactamases have been widely studied. Several primary structures are known and the three-dimensional structures of some β-lactamases have been solved at high resolution [8–10]. β-lactamases are, therefore, appropriate candidates for a detailed comparison of the functional and structural properties of a psychrophilic enzyme with its mesophilic counterparts. β-lactamases are commonly classified into four groups. Class A (penicillinases), class C (cephalosporinases) and class D enzymes belong to the active-serine enzyme superfamily and differ at the level of their primary structure, whereas class B β-lactamases rely on the presence of a Zn^{2+} in their active site.

MATERIALS AND METHODS

Sources. *P. immobilis* A5 was isolated from frozen organic debris in Terre Adelie near the antarctic station Dumont d'Urville (66° 40′ S, 140° 01′ E). The class C β-lactamase from the mesophile *Enterobacter cloacae* Q908R was a gift from Prof. J. M. Frère (University of Liège). Manufacturers of the β-lactam compounds are those previously cited [11, 12].

β-lactamase purification. The strain was cultivated at 4°C for 4 days in 3 l of TYK medium containing 16 g/l bactotryptone, 5 g/l yeast extract, 2.5 g/l K_2HPO_4, pH 7.6. After centrifugation at 23000 *g*, the culture supernatant was concentrated to 300 ml and diafiltered against 25 mM Tris/HCl, pH 8.0, by using a Minitan tangential flow ultrafiltration unit (Millipore) fitted with PTGC membranes (10-kDa retention limit). The sample was loaded on a DEAE-cellulose column (3.0 cm×25 cm) equilibrated in the above mentioned buffer and eluted with a NaCl linear gradient (220 ml to 220 ml, 0 to 0.2 M NaCl) applied after

Table 1. Purification of the extracellular β-lactamase from *P. immobilis* A5.

Purification step	Volume	Protein	Specific activity	Yield	Purification
	ml	mg	μmol · min^{-1} · mg^{-1}	%	-fold
Culture supernatant	2800	538	0.9	100	1.0
Diafiltration	290	239	1.0	85	1.1
DEAE-cellulose	34	21.2	8.5	65	9.5
Sephacryl S-200	56	5.3	31.1	51	34.6
Phenylboronic-acid−agarose	20	0.06	2094	24	2327

100 ml buffer elution. Fractions containing the β-lactamase activity were concentrated to 10 ml and applied onto a Sephacryl S-200 column (2.5 cm×100 cm) eluted with 25 mM Tris/HCl, pH 8.0. The active fractions were adjusted to 0.5 M NaCl and concentrated to 5 ml. The sample was then loaded on a phenylboronic-acid−agarose column (1.5 cm×20 cm) prepared according to [13] and eluted with 25 mM Tris/HCl, 0.5 M NaCl, pH 8.0. To elute the enzyme in a sharp peak, 125 mM $K_2B_4O_7 \cdot 4\ H_2O$ was added to the irrigating buffer after 24 ml elution.

Enzyme assay and kinetic parameters. Standard assay of β-lactamase activity was carried out at 25°C using 300 μM nitrocefin as substrate in 50 mM sodium phosphate, pH 7.5, containing 100 μg/ml BSA. Activities towards the chromogenic substrate were recorded in a temperature-regulated Uvikon 860 spectrophotometer (Kontron) and were calculated using an absorption coefficient of 15000 M^{-1} cm^{-1} at 482 nm [14]. The values of k_{cat} and K_m for nitrocefin and for cefazolin were measured by analysing the complete progress curves of the reaction [11]. For cephalexin, benzylpenicillin and ampicillin, K_m values were measured as K_i by using 100 μM nitrocefin as the reporter substrate [12], and k_{cat} values were obtained with saturating concentrations of substrate.

Analytical procedures. Protein concentrations were determined using the Coomassie protein reagent (Pierce). SDS/PAGE and isoelectric focusing were run essentially as described by the supplier of the electrophoretic equipment (Hoeffer Scientific Instruments). The N-terminal amino acid sequence of the native enzyme was determined using a pulsed-liquid-phase protein sequenator (Applied Biosystem 477A) equipped with an on-line 120A phenylthiohydantoin analyzer.

Cloning and sequencing the *ampC* gene. Genomic DNA of *P. immobilis* A5 was partially digested with *Sau*3AI and the resulting fragments were inserted into the single *Bam*HI site of pBGS18$^+$ [15]. The ligated DNA fragments were electroporated in *Escherichia coli* RR1 cells and the transformants were selected on Luria-Bertani agar plates containing 50 μg kanamycin/ml. The temperature of growth and incubation was controlled as previously described [16]. Plates were sprayed with a sterile solution of nitrocefin. Among 3000 transformants screened, one clone developed a red halo corresponding to the hydrolysis of nitrocefin. The *ampC*-gene-containing DNA fragment was subcloned into the polylinker of the phagemids pGEM3Zf$^+$ and f$^-$. Single-stranded DNA was obtained by infecting pGEM3Zf-transformed *E. coli* JM109 cells with the helper phage M13K07, inducing the bacteriophage f1 replication (pGEM Single Strand System, Promega). The nucleotide sequence of both strands was determined using Sequenase (US Biochemical Corp.).

Molecular modelling. The model for the structure of *P. immobilis* β-lactamase was built using the homology modelling program COMPOSER [17, 18], starting from the atomic coordinates of *E. cloacae* cephalosporinase (Brookhaven Protein Data Bank, filename 1BLT). The structure was minimized within the AMBER molecular mechanics V4.1 framework [19] in order to release some short contacts. Both programs were run on a Silicon Graphics Indy workstation.

RESULTS

β-lactamase production and purification. The antarctic bacterial strain *P. immobilis* A5 is able to grow between 0°C and 25°C. However, optimal growth yield and β-lactamase secretion were found below 5°C. The effects of temperature on growth and on enzyme secretion of *P. immobilis* and of some other antarctic bacteria have been described elsewhere [20]. β-lactamase secretion into the culture supernatant followed bacterial growth and about 15% of the total activity remained cell associated at the early stationary phase. No induction of β-lactamase production was recorded following addition of 10 μg/ml benzylpenicillin, Δ^3-desacetoxy-7-aminocephalosporonate or 6-aminopenicillinic acid whereas 5 g/l NaCl inhibited 50% of the enzyme production.

The β-lactamase was purified from culture supernatant of *P. immobilis* grown at 4°C, using a phenylboronic-acid−agarose column at the final step (Table 1). Because the enzyme is not bound to this affinity medium (but is only retarded), two other chromatographic steps were required in order to remove contaminating peptides and pigments. About 20 μg β-lactamase were purified from 1 l culture; SDS/PAGE revealed a single band and no significant contamination was detected by N-terminal amino acid sequencing. According to the purification yield (24%), β-lactamase secretion in the supernatant was estimated to 85 μg/l.

Characterization of *P. immobilis* β-lactamase. The molecular mass of the native β-lactamase was found to be 41 kDa by SDS/PAGE and its pI to be 5.4 as indicated by isoelectric focusing under non-denaturing conditions. The activity of *P. immobilis* β-lactamase is unaffected by 5 mM EDTA indicating that it does not belong to the class B metalloenzymes. By contrast, incubation at 4°C in the presence of 10 μM oxacilline results in a complete inhibition of the activity towards nitrocefine. Kinetic parameters for the hydrolysis of three cephalosporins (nitrocefin, cephalexin and cefazolin) and two penicillins (benzylpenicillin and ampicillin) have been determined. As shown in Table 2, cephalosporins are good substrates for *P. immobilis* β-lactamase. This catalytic profile is similar to that determined for other class C β-lactamases from mesophilic bacteria [11, 12].

Stability and thermodependence of activity. The enzyme is stable in the high pH range with an optimum value near pH 8.5 (Fig. 1A). Inactivation at neutral and low pH suggests that protonation of histidyl residues destabilizes the psychrophilic enzyme. The denaturation curves of β-lactamases from *P. immobilis* and from the mesophilic *E. cloacae* clearly illustrate the low thermal stability of the psychrophilic enzyme (Fig. 1B). The half-time for inactivation of the psychrophilic β-lactamase at

Table 2. Kinetic parameters for the hydrolysis of some β-lactam antibiotics by the psychrophilic and mesophilic class C β-lactamases. All data have been recorded at 30°C. SD ≤10% of the values reported for the psychrophilic enzyme. Data for the mesophilic enzymes are from [11, 12].

Substrate	*P. immobilis* β-lactamase			Mesophilic class C β-lactamases		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$s^{-1}\ \mu M^{-1}$	s^{-1}	μM	$s^{-1}\ \mu M^{-1}$
Nitrocefin	1407	51	27.6	330 −1240	12 − 500	1.0−34
Cephalexin	26	8	3.3	27 − 150	3 − 30	2.7−12.5
Cefazolin	476	100	4.8	150 −5000	400 −1500	0.4− 8.0
Benzylpenicillin	20	1.7	11.8	14 − 76	0.4 − 4.4	10 −75
Ampicillin	1	6	0.16	0.5− 6.5	0.01− 3.5	1.2−46

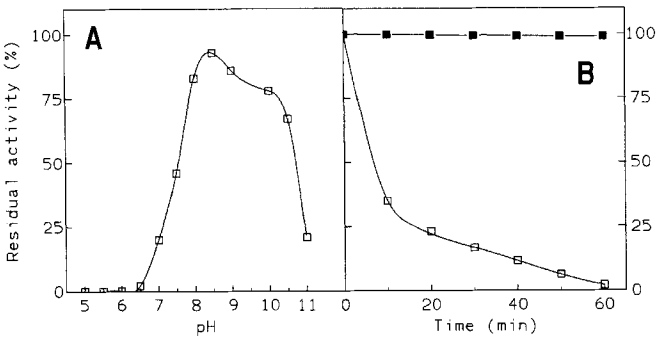

Fig. 1. Stability of *P. immobilis* β-lactamase. (A) pH stability recorded after 24 h incubation at 4°C in 25 mM each acetate, Pipes, Tris and Caps at the indicated pH values. Residual activities were recorded using nitrocefin as substrate. (B) Thermal stability of β-lactamase from *P. immobilis* (□) and *E. cloacae* Q908R (■) at 50°C in 25 mM Tris/HCl, pH 8.5, containing 100 μg/ml BSA.

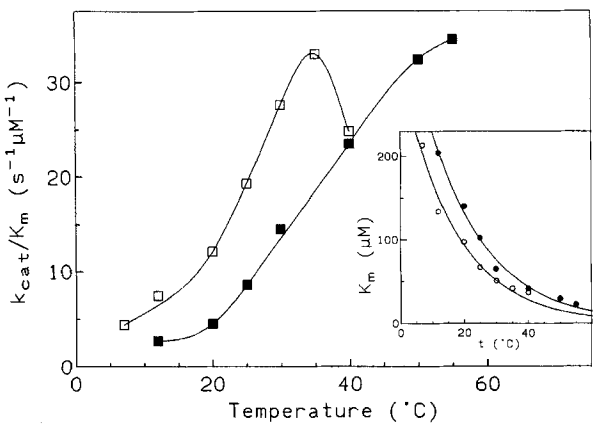

Fig. 2. Temperature dependence of the nitrocefin hydrolysis. Kinetic parameters of β-lactamase from *P. immobilis* (□) and *E. cloacae* Q908R (■) for the hydrolysis of nitrocefin were recorded at increasing temperatures as described in the Materials and Methods section.

50°C is about 7 min whereas the activity of the mesophilic enzyme remains unaltered for more than 1 h in these conditions. Fig. 2 illustrates the effect of temperature on the kinetic parameters of β-lactamases from the psychrophilic and mesophilic strains. The thermodependence curves of the k_{cat}/K_m values display a shift of the apparent optimal temperature of activity of about 20°C towards lower temperatures in the case of the psychrophilic enzyme. As the decrease in K_m values with temperature is similar for both enzymes (Fig. 2), this difference mainly arises from the thermodependence of k_{cat}.

Sequence of the chromosomal *ampC* gene. The structural gene of the β-lactamase was cloned from a genomic library of *P. immobilis* in *E. coli*. To circumvent thermal denaturation of the cloned gene products, *E. coli* transformants were allowed to grow overnight at 25°C. When sprayed with nitrocefin, one clone produced a red halo of substrate hydrolysis, demonstrating the expression of an active recombinant β-lactamase. The 14-kb cloned DNA fragment was also found to confer ampicillin resistance (50 μg/ml) to the host strain in addition to the plasmid-encoded kanamycin resistance. Taking advantage of the latter property, the structural *ampC* gene was located on a 2.4-kb *Sph*I−*Sau*3AI restriction fragment which was sequenced on both strands. The primary structure of the β-lactamase presursor from *P. immobilis* was deduced from the nucleotide sequence and is shown in Fig. 3. Determination of the N-terminal amino acid sequence of the purified native enzyme indicates that a peptide made of 39 amino acid residues is cleaved from the β-lactamase precursor. However, a potential signal-peptidase-cleavage site which fulfils the −3, −1 rule [21] is also found 23 residues downstream from the N-terminal formylmethionine (Fig. 3), suggesting that the β-lactamase precursor has the structure of a preproenzyme. The mature *P. immobilis* β-lactamase is composed of 362 amino acid residues with a predicted molecular mass of 40321 Da, in good agreement with the electrophoretic estimation. Boxed in Fig. 3 are the four structural and functional elements conserved in β-lactamases [22], supplying most of the groups involved in the catalysis and in the active-site architecture [9, 10, 23, 24]. The active-site serine is found at position 63 in the first conserved element. Sequence alignment with six other class C β-lactamases (Fig. 4) indicates that the *P. immobilis* enzyme possesses between 38% (*E. coli*) and 43% (*Pseudomonas aeruginosa*) amino acid sequence identity when compared with β-lactamases from mesophilic bacteria.

Structural analysis of *P. immobilis* β-lactamase. Striking similarities in the general folding have been noted in the crystal structures of two solved class C β-lactamases [23, 24]. This allowed the building of a three-dimensional model of the psychrophilic enzyme based on *E. cloacae* β-lactamase atomic coordinates at 0.2-nm resolution (coordinates of the *Citrobacter freudii* enzyme are not available). The rmsd for the Cα atoms from both structures was 0.127 nm, with the largest differences observed in the loop between helices α5 and α6 where two proline residues are lacking in *P. immobilis* β-lactamase. The molecular architecture follows the pattern of the known β-lactamases structures with an all-helical domain and a mixed α/β domain; the

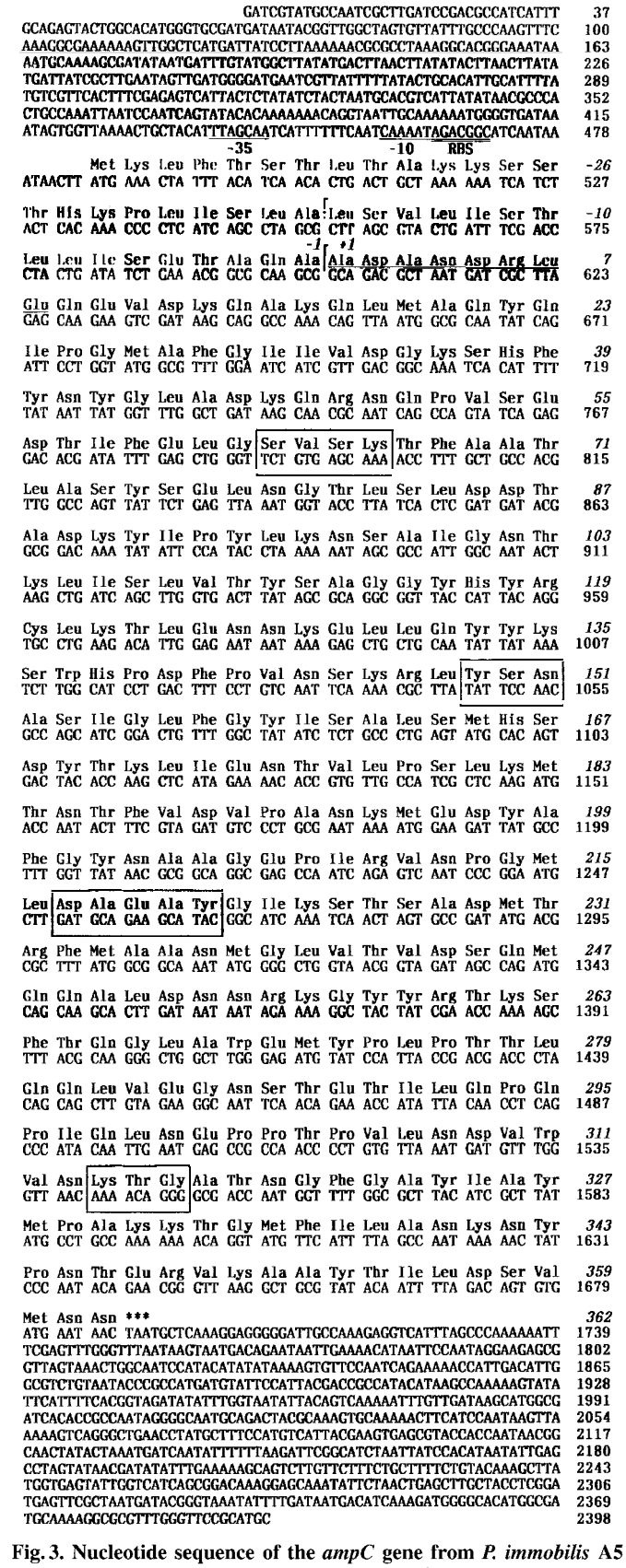

Fig. 3. Nucleotide sequence of the *ampC* gene from *P. immobilis* A5 and the deduced amino acid sequence of the β-lactamase precursor. Amino acids are numbered starting at the N-terminal residue of the native enzyme determined by amino acid sequencing (underlined). The consensus signal peptidase cleavage site is located by a dashed bracket. The four conserved elements of β-lactamases are boxed; the potential promoter regions (−35,−10) and ribosome-binding site (RBS) are also indicated.

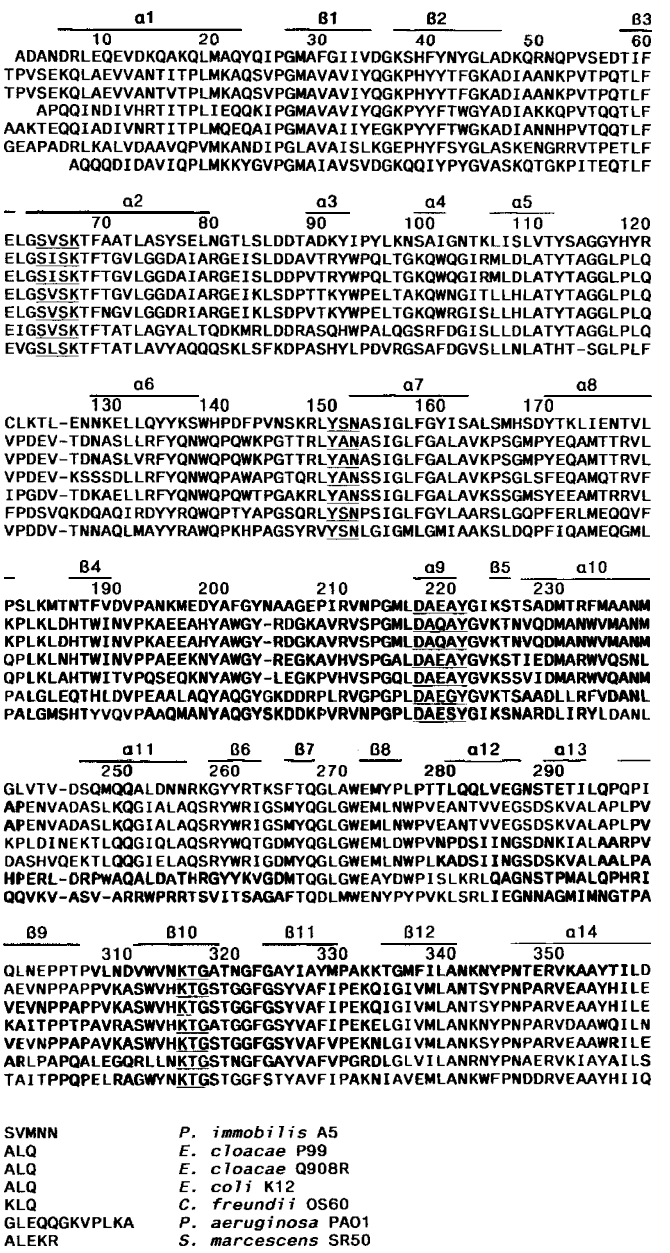

Fig. 4. Amino acid sequence alignment of class C β-lactamases. Sequences of class C β-lactamases have been aligned using the Pileup program (GCG). The numbering corresponds to the *C. freundii* enzyme. The secondary structures of *E. cloacae* P99 β-lactamase [24] are indicated and the four conserved elements are underlined.

active-site serine is located in a cleft between the two domains at the N-terminus of the α2 helix. The crystal structure of the *E. cloacae* enzyme and the three-dimensional model of *P. immobilis* β-lactamase were analysed in order to identify the weak interactions and the structural features potentially involved in the low stability and structure flexibility of the psychrophilic enzyme. The results are discussed below.

DISCUSSION

All pathogenic gram-negative bacteria producing β-lactamases concentrate the enzyme in the periplasmic space where the penicillin targets are located. By contrast, *P. immobilis* A5 secretes its β-lactamase to the culture medium. As already mentioned for a closely related strain [7], β-lactamase secretion is

probably an ancestral, but weakly efficient, mechanism of protection against bacteriolytic microoganisms. It should be noted that the *P. immobilis* β-lactamase precursor seems to be composed of a preproenzyme. The small potential propeptide (16 amino acid residues) following the consensus signal peptide (Fig. 3) is possibly involved in the extracellular secretion pathway.

The various structural and kinetic properties of the psychrophilic enzyme relates it to the well-characterized class C β-lactamases. *P. immobilis* β-lactamase displays a low apparent optimal temperature of activity and a low thermal stability which are common characteristics of proteins from cold-adapted organisms [5, 6]. However, the high specific activity usually recorded, which is the main adaptation to catalysis at low environmental temperatures [25, 26], is not clearly observed. The temperature dependence of nitrocefin hydrolysis (Fig. 2) marginally favours the psychrophilic β-lactamase at low temperatures and the kinetic parameters for the hydrolysis of penicillins and cephalosporins (Table 2) are similar to the values determined for mesophilic strains. It should be noted that the comparison is made with highly specialized cephalosporinases from pathogenic bacteria that have evolved under the strong selective pressure of antibiotics: they are themselves a special class of extremophiles. However, the surrounding concentration of β-lactam antibiotics in the antarctic environment is probably very low and the enzyme could be highly specific to an as-yet unidentified substrate.

The model structure of *P. immobilis* β-lactamase provides insights into the molecular origin of heat lability. This enzyme possesses the lowest proline content (18 versus 22−27 residues) and arginine content (9 versus 11−24 residues) when compared with other class C enzymes. Proline contributes to the local rigidity of the peptidic backbone because the pyrrolidine ring severely restricts the available dihedral angles of the preceding residue and decreases the backbone entropy of unfolding. When compared with mesophilic β-lactamases, substitutions for non-proline residues in the *P. immobilis* enzyme occur mainly in loops connecting secondary structures. Three proline residues are strictly conserved in all mesophilic class C β-lactamases but are replaced in the psychrophilic enzyme. The substitution Pro18→Gln (*C. freundii* numbering) avoids a bend in the N-terminal, solvent-exposed helix α1; substitutions Pro188→His and Pro122→Leu increase the flexibility of the loop between helices α5 and α6 at the entrance of the active site. A structural role for arginine through multiple hydrogen bonds to backbone carbonyl and side-chain oxygens has been proposed [27]. For instance, we noted a typical structural arginine conserved in class C β-lactamases in position 349 and forming all the five hydrogen bonds allowed by the guanidinium group. Inspection of the crystal structure of *E. cloacae* β-lactamase reveals 16 arginine-mediated hydrogen bonds whereas only 10 bonds are found in the heat-labile *P. immobilis* enzyme. Nine weakly polar interactions between aromatic rings have been recorded in the mesophilic β-lactamase structure using the parameters given by Burley and Petsko [28]. These interactions are strongly conserved in all class C enzymes underlining their critical role in β-lactamase conformation. However, two interactions involving Trp101 (with Tyr135 and Trp138) and Trp276 (with Tyr354) are specifically lacking in *P. immobilis* β-lactamase. Chromosomally encoded class C β-lactamases from mesophilic bacteria in general are basic proteins but the psychrophilic enzyme displays an acidic pI arising from 5−6 extra acidic side chains. Similar discrepancies in pI values have been recorded between a psychrophilic subtilisin and its mesophilic or thermophilic counterparts [25]. In that case, it was expected that improved solvent interactions through additional surface acidic residues can destabilize the external shell of the protein. The clustering of these solvent-exposed amino acids within the first 30 residues of *P. immobilis* β-lactamase and the lack of a conserved proline in helix α1 would reduce the packing (or contacts) of the N-terminal extremity in the α/β domain. The hydrophobic effect is a well-recognized determinant of folding stability [29]. Ikai [30] has correlated an aliphatic index, calculated from the molar ratio and the relative volume of Ala, Val, Ile and Leu, with the stability of thermophilic proteins. This index frequently reaches 90−100 in thermophiles and ranges between 81−86 in mesophilic class C β-lactamases; the aliphatic index decreases to 77 for the the cold-adapted enzyme suggesting a lower global hydrophobicity. In addition, the prefered amino acid exchanges have been determined by exchange matrixes according to [31, 32]. The most-favoured residue exchanges from the psychrophilic to the mesophilic β-lactamases were Asn→Gly, Arg, Gln; Ser, Gln→Ala; Lys→Arg; Met, Val→Leu. All the substitutions decrease the hydrophobicity of the antarctic enzyme when using the PRIFT scale [33], except the Asn→Gln replacement. The same conclusion applies when the statistical analysis is restricted to α-helices, with Ser, Gln→Ala being the preferred exchanges. These substitutions also decrease the flexibility index and the α-helix-forming ability of individual amino acids.

The structure of *E. cloacae* β-lactamase is stabilized by relatively few salt bridges: two ion pairs bonding distant parts of the molecule (Glu82−Arg177 and Arg105−Glu300) and a cluster of electrostatic interactions (His186−Glu195, Lys193−Glu196 and Glu195−His198). Only one salt bridge (Lys195−Asp198) is found in the psychrophilic enzyme. One should note however that *P. aeruginosa* and *Serratia marcescens* β-lactamases also lack these bonds and therefore no conclusions can be drawn. No significant differences were observed in charge-dipole interactions within α-helices of the class C β-lactamases.

In conclusion, several structural factors and weak interactions likely to promote enzyme stability are specifically lacking in *P. immobilis* β-lactamase. The disappearance of these stabilizing elements affect all regions of the enzyme molecule, rather than a specific domain, and seems to be involved in the marked heat lability and the expected conformational flexibility of the cold-adapted β-lactamase. It is worth mentioning that the same factors have been implicated in the stability of thermophilic proteins, i.e. an increase of proline residues in loops [34, 35] and of the Arg molar ratio [36], the reinforcement of aromatic-aromatic interactions [37] and of the hydrophobic effect [38], the occurrence of several salt-bridged surface residues [39, 40], interactions preventing unzipping of peptidic terminii [41] and a statistical tendency to increase hydrophobicity, rigidity and α-helix-forming ability of residues in α-helices [31, 32]. Obviously, the required compromise between enzyme activity at extreme temperatures and the molecular dynamic (flexibility/rigidity) is reached through the same adaptational strategies.

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