

Crystal structure of a cold-adapted class C β -lactamase

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Database

The protein sequence has been deposited in the UniProt Knowledgebase (P85302) with Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC 20007

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, under the code 2QZ6 (<http://www.rcsb.org/>)

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In this study, the crystal structure of a class C β -lactamase from a psychrophilic organism, *Pseudomonas fluorescens*, has been refined to 2.2 Å resolution. It is one of the few solved crystal structures of psychrophilic proteins. The structure was compared with those of homologous mesophilic enzymes and of another, modeled, psychrophilic protein. The elucidation of the 3D structure of this enzyme provides additional insights into the features involved in cold adaptation. Structure comparison of the psychrophilic and mesophilic β -lactamases shows that electrostatics seems to play a major role in low-temperature adaptation, with a lower total number of ionic interactions for cold enzymes. The psychrophilic enzymes are also characterized by a decreased number of hydrogen bonds, a lower content of prolines, and a lower percentage of arginines in comparison with lysines. All these features make the structure more flexible so that the enzyme can behave as an efficient catalyst at low temperatures.

β -Lactamases are the major causes of bacterial resistance to the β -lactam family of antibiotics, such as penicillins and cephalosporins. These enzymes catalyze

the hydrolysis of the critical β -lactam ring and render the antibiotic inactive against its original cellular target, the cell wall transpeptidase. β -Lactamases of

Abbreviations

F_0 , F_t and F_∞ , fluorescence intensities at $t = 0$, $t = t$ and $t = \infty$, respectively; F_N and F_D , intrinsic fluorescence of the native and denatured form, respectively; F_{obs} , observed fluorescence; k_d , denaturation rate constant; m_{D-N} , slope of the line relating the free energy difference between the native (N) and denatured (D) form at a given urea concentration to the urea concentration; ΔG^0_{D-N} , free energy difference between the native (N) and the denatured (D) form without denaturing agent.

classes A, C and D are active site serine enzymes, whereas class B β -lactamases require one or two zinc ions for their activity [1]. Only class C β -lactamases were found to be synthesized by ampicillin-resistant psychrophilic bacteria collected in the Antarctic [2].

Psychrophilic strains, and particularly their enzymes, have generated considerable interest and have been proposed for a number of applications in fundamental research [3,4], in biotechnology to improve the efficiency of industrial processes, and for environmental applications [5–7].

‘Cold enzymes’ from psychrophilic microorganisms are generally characterized by a higher catalytic activity and efficiency ($k_{\text{cat}}/K_{\text{m}}$) at low temperatures than their mesophilic counterparts [8]. The ability of psychrophilic microorganisms to survive and proliferate at low temperatures implies that they have overcome key barriers inherent to permanently cold environments, such as protein cold-denaturation, inappropriate protein folding, and reduced enzyme activity, to name a few [9]. The commonly accepted hypothesis for this cold adaptation is the activity–stability–flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structures to compensate for the ‘freezing effect’ of cold habitats [8,10–14]. Increased intramolecular flexibility is achieved through weakening of interactions that stabilize the native protein molecules, especially those involved in catalysis, with a concomitant reduction in stability of cold-adapted enzymes [15,16].

A general theory for cold adaptation has not been formulated yet, as different enzymatic families can follow different evolutionary strategies. Therefore, recently, the research community has focused on comparative structural investigations of homologous proteins adapted to different temperature conditions [17–25]. In contrast to thermophilic proteins, few crystal structures have been solved for psychrophilic proteins, probably because their thermolability and flexibility result in handling and crystallization difficulties [26].

Analysis of the available 3D structures and site-directed mutagenesis experiments has shown that the low stability of cold-adapted enzymes has been achieved through: a reduction of the number and/or strength of weak interactions; increased interactions with the solvent; a decrease in the number and/or strength of hydrophobic internal clusters; and entropic effects tending to increase the entropy of the unfolded form and to lower its free energy. Each cold-adapted enzyme is modulated using a specific strategy, probably as a function of structural requirements, and makes a selection among the above-mentioned factors

to improve the flexibility at the level of the catalytic site [27].

In this work, we describe the crystal structure of a psychrophilic class C β -lactamase from *Pseudomonas fluorescens* TAE4 [2] and compare its structure to those of three homologs produced by the psychrophile *Psychrobacter immobilis* [28] and the two mesophiles *Enterobacter cloacae* 908R and *Serratia marcescens* [29]. These enzymes were selected because of their availability for experimental assays. The 3D structure of the homologs was modeled, as no structure was available in the Protein Data Bank, except for the mesophile 908R (Protein Data Bank entry 1Y54). The comparison of these structures of psychrophilic enzymes with those of mesophilic counterparts with high sequence identity provides further insights into the understanding of cold adaptation.

Results

Kinetic characterization of the cold enzyme from *Pse. fluorescens* TAE4

Kinetic parameters for the hydrolysis of three cephalosporins (nitrocef, cephalixin, and cefazolin) and five penicillins (benzylpenicillin, ampicillin, carbenicillin, oxacillin, and cloxacillin) were determined for the TAE4 β -lactamase and compared with those of the enzymes from *Psy. immobilis*, *E. cloacae* 908R and *S. marcescens* (Table 1). The substrate profile of the TAE4 β -lactamase is globally similar to that of its psychrophilic and mesophilic homologs, except for penicillins with larger side chains (oxacillin and cloxacillin) and carbenicillin. The latter are very poor substrates of mesophilic class C β -lactamases. The k_{cat} values measured for *Pse. fluorescens* are 26–130 times higher than those of *E. cloacae* 908R. As the K_{m} values are also higher (lower apparent affinity), the $k_{\text{cat}}/K_{\text{m}}$ ratios are similar for both enzymes. These data probably result from a difference in the deacylation rates between the enzymes.

Stability and thermal and urea denaturation of the cold enzyme from *Pse. fluorescens* TAE4

Thermal inactivation of β -lactamases from *Pse. fluorescens*, *Psy. immobilis* A5, *S. marcescens* and *E. cloacae* was studied at one or different temperatures following fluorescence quenching (Table 2). The thermal denaturation is irreversible for the four proteins, and therefore only kinetic parameters can be deduced. Both cold enzymes (*Pse. fluorescens*, *Psy. immobilis*) are more sensitive to thermal denaturation than their mesophilic

Table 1. Kinetic parameters (at 30 °C) for the hydrolysis of cephalosporins and penicillins by the psychrophilic and mesophilic β -lactamases.

Substrate	<i>Pse. fluorescens</i> TAE4			<i>Psy. immobilis</i> ^a			<i>E. cloacae</i> 908R			<i>S. marcescens</i>		
	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)
Nitrocefin	550 ^b	116 ^b	4.75	1407	51	27.6	780 ^e	23 ^e	34 ^e	1240 ^e	40 ^e	30 ^e
Cephalexin	31 ^c	50 ^c	0.63	26	8	3.3	72 ^e	8.5 ^e	8.5 ^e	80 ^e	30 ^e	2.7 ^e
Cefazolin	> 61 ^b	> 150 ^b	0.41	476	100	4.8	3000 ^e	1500 ^e	2 ^e	1300 ^e	540 ^e	2.4 ^e
Benzylpenicillin	34.3 ^c	2.3 ^d	34.3	20	1.7	11.8	18 ^f	0.5 ^f	36 ^f	75 ^f	1.7 ^f	44 ^f
Ampicillin	0.65 ^c	5.1 ^d	0.65	1	6	0.16	0.53 ^f	0.4 ^f	1.3 ^f	0.46 ^f	0.01 ^f	46 ^f
Carbenicillin	0.52 ^c	3.7 ^d	0.14	—	—	—	0.004 ^f	0.004–0.011 ^f	0.37 ^f	—	—	—
Oxacillin	0.21 ^c	0.118 ^d	1.78	—	—	—	0.008 ^f	0.0006–0.0011 ^f	7 ^f	—	—	—
Cloxacillin	0.42 ^c	0.016 ^d	27.1	—	—	—	0.004 ^f	0.0005 ^f	9 ^f	—	—	—

^a Data from [38]. ^b Complete time-courses [42]. ^c Initial rates. ^d Substrate competition. ^e Data from [43]. ^f Data from [54].**Table 2.** Thermal denaturation rate constant, k_d (s⁻¹), determined by fluorescence. ND, not determined.

	T (°C)		
	50	55	60
<i>Pse. fluorescens</i>	4.8 \pm 0.2	19.0 \pm 1.5	43.0 \pm 0.6 ^a
<i>Psy. immobilis</i>	13.3 \pm 1.3	47 \pm 2	ND
<i>E. cloacae</i>	ND	ND	33 \pm 3
<i>S. marcescens</i>	0.22 \pm 0.02	ND	21 \pm 2

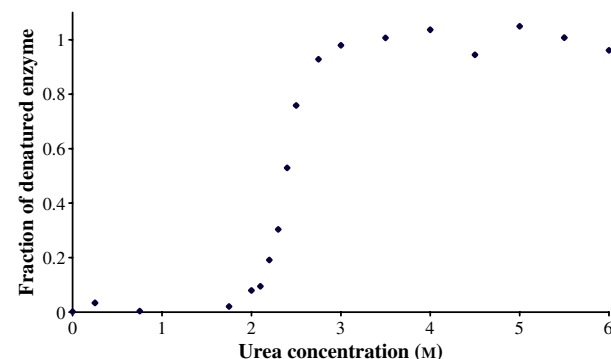
^a Determined at 57.5 °C.

homologs. At 50 °C, the measured k_d values for both Antarctic enzymes are 22–60 times larger than that of *S. marcescens*.

Intrinsic fluorescence of the psychrophile TAE4 was also measured as a function of the urea concentration at 30 °C (Fig. 1). As denaturation of *Pse. fluorescens* TAE4 by urea is nearly fully reversible (more than 95%), thermodynamic parameters can be deduced. The C_m , the slope of the line relating the free energy difference between the native (N) and denatured (D) form at a given urea concentration to the urea concentration (m_{D-N}) and the free energy difference between the native (N) and the denatured (D) form without denaturing agent (ΔG^0_{D-N}) were 2.4 M urea, 3.7 kcal·mol⁻¹·M⁻¹ and 8.7 kcal·mol⁻¹, respectively. The thermodynamic stability of TAE4 is lower than that of the mesophilic enzyme, AmpC, as the ΔG^0_{D-N} value is 5.3 times smaller for the cold enzyme [30].

Sequence comparison

The complete amino acid sequence of the psychrophile TAE4 was determined using analyses carried out on the protein itself. With exception of the dipeptide Leu83-Lys84, which was lost during purification of the

**Fig. 1.** Intrinsic fluorescence of the *Pse. fluorescens* TAE4 as a function of the urea concentration at 30 °C.

Lys-C protease-generated peptides, all other amino acids could be identified in at least one of the peptides generated by the three proteases used. The summed molecular masses of the subsequent Lys-C peptides was 38 720.2 Da, which agrees with the experimentally determined mass of the protein of 38 723.1 Da (\pm 4.9 Da). The converted spectrum reveals a shoulder at a mass of around 38 700 Da, which reflects a one-residue heterogeneity detected by chemical C-terminal sequence analysis (-SAMDQ and -SAMD).

The four studied β -lactamases, aligned using CLUSTALW, share an amino acid sequence identity of about 40–50% (Fig. 2 and Table 3). The C-terminal region is relatively conserved, whereas the N-terminal region is the most variable (data not shown). They share the three characteristic motifs of serine-reactive β -lactamases [31,32]: S-X-X-K, with Ser64 and Lys67 forming hydrogen bonds in the active site, Y-X-N, with Tyr150 and Asn152 pointing into the active site, and KTG (Lys315), forming the opposite wall of the active site

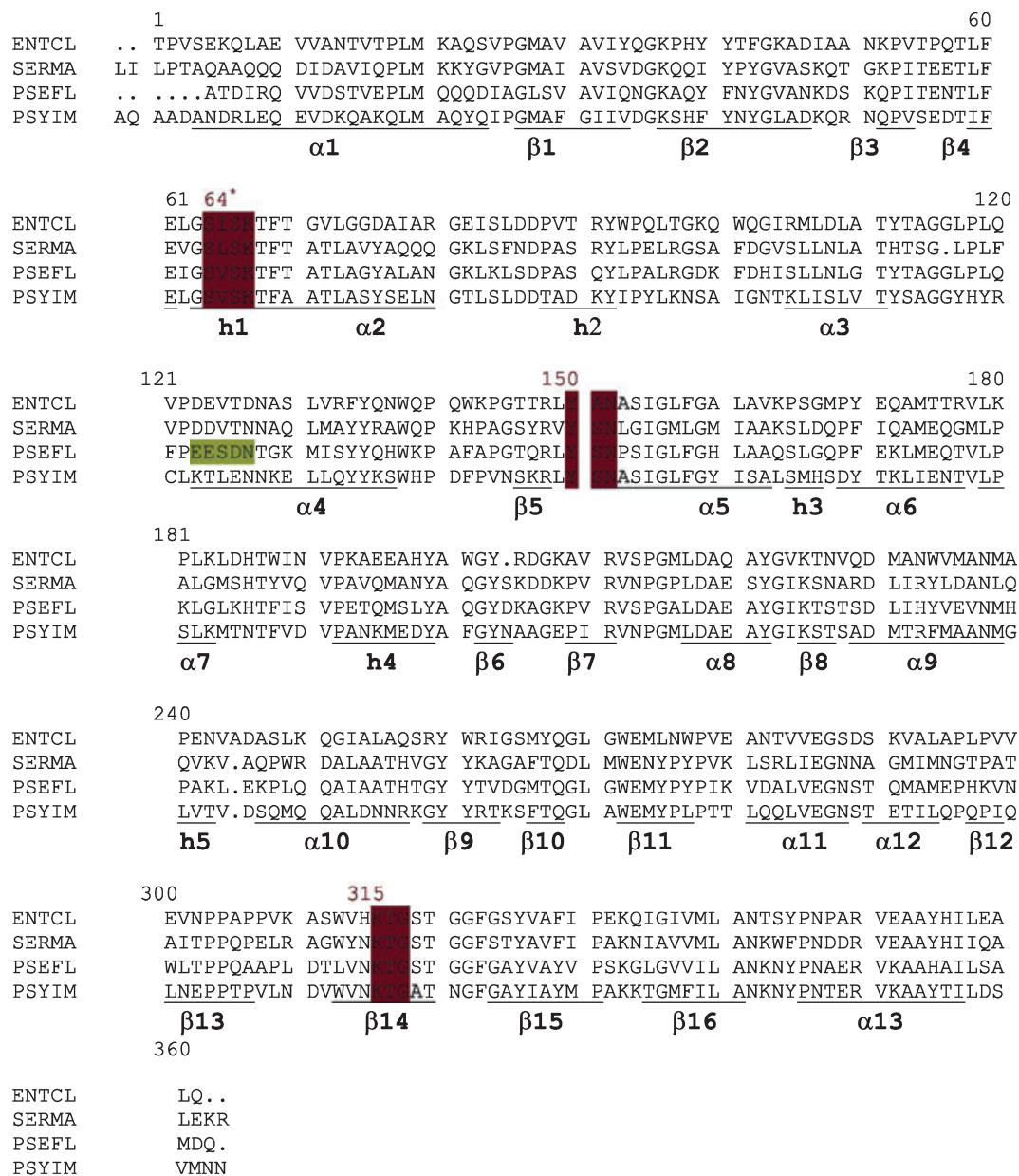


Fig. 2. Sequence alignment of class C β -lactamases from *Pse. fluorescens* (PSEFL), *Psy. immobilis* (PSYIM), *E. cloacae* (ENTCL) and *S. marcescens* (SERMA). The three motifs characteristic of active site serine β -lactamases are in red. The disordered sequences of PSEFL are in green.

Table 3. Percentage identity between the four β -lactamases and rmsd values (\AA) for C_{α} atoms among the four studied enzymes. Parameters potentially involved in thermal adaptation. ASA, accessible surface area.

	<i>Pse. fluorescens</i>	<i>Psy. immobilis</i>	<i>E. cloacae</i>	<i>S. marcescens</i>
Percentage identity [rmsd (\AA)] with <i>Pse. fluorescens</i>	–	47 (0.77)	47 (0.79)	51 (0.75)
Percentage identity [rmsd (\AA)] with <i>Psy. immobilis</i>	–	–	37 (0.35)	40 (0.15)
Percentage identity [rmsd (\AA)] with <i>E. cloacae</i>	–	–	–	42 (0.32)
Temperature adaptation	Psychrophile	Psychrophile	Mesophile	Mesophile
Protein Data Bank code/template (% identity ^a)	2QZ6	Model (1FR1) (35.6)	1Y54	Model (1XX2) (41.3)
% (D + E + K + R)	16	18	16	16
%R	1.40	2.24	3.15	3.44
%K	6.4	6.7	5.2	5.3
K/R	4.60	3.00	1.67	1.5
%(A + I + L + V)	30.9	30	33	33
%(F + Y + W)	9	10	9	9.5
%P (No.)	6.7 (24)	4.7 (19)	7.3 (28)	6.9 (26)
%G	8.7	5.7	8.1	7.4
%M	2.8	3.7	1.5	4
Hydrophobic contacts	435	488	376	517
No. ion pairs	3	3	11	Asp103–Arg107
	Glu245–Lys181	Asp85–Arg88	Glu5–His39	Asp251–Lys258
	Glu236–His240	Asp73–Lys75	Glu195–His186	Asp283–His271
	Glu272–Arg148	Glu310–Arg186	Glu195–His198	Glu366–His370
			Glu358–His355	Glu287–Arg163
			Asp76–Arg80	
			Glu82–Arg177	
			Asp108–Arg105	
			Asp185–Lys183	
			Glu196–Arg210	
			Glu300–Arg105	
			Glu272–Arg148	
No. of hydrogen bonds (side chain–side chain)	19	15	21	21
Aromatic stacking	19	21	19	21
ASA total (\AA^2)	14 092	15 880	14 561	15 379
ASA apolar (\AA^2)	8248	8931	8572	9059
ASA polar (\AA^2)	5844	6949	5990	6320
Formal global charge	–1	–3	1	2
Theoretical pI ^b	6.5	7	8.5	9

^a Following CLUSTALW program. ^b Calculated from COMPUTE PI/MW (http://www.expasy.ch/tools/pi_tool.html).

(Fig. 2). The enzymes therefore exhibit all the properties common to class C β -lactamases, but are adapted to different temperatures, and therefore constitute an adequate series of homologous enzymes for temperature adaptation studies. Composition analysis shows that the psychrophilic enzymes have a slightly lower arginine and a higher lysine content than their mesophilic homologs (Table 3). The contents of other charged residues are similar in both kinds of enzymes. Several glycines and prolines are conserved in the four enzymes, but the number of prolines is smaller in the psychrophilic enzymes, the lowest number being found in *Psy. immo-*

bilis. Whereas the content of hydrophobic residues with aromatic rings is similar, some differences are observed in alanine, isoleucine, valine and leucine contents. Globally, the mesophilic enzymes have slightly more hydrophobic residues than their psychrophilic homologs.

Crystal structure of β -lactamase TAE4 from *Pse. fluorescens* and structure comparison with related enzymes

Crystals of the β -lactamase TAE4 belong to space group P2₁, with unit cell parameters $a = 43.6$,

$b = 69.7$, $c = 53.9$ Å, and $\beta = 90.9^\circ$. The crystal structure of the enzyme was determined by the molecular replacement method, based on the structure of the class C β -lactamase from *E. cloacae* P99 (Protein Data Bank code 2BLT) as a search model. The model was solved to a resolution of 2.2 Å. A summary of data collection and refinement statistics is presented in Table 4. Ramachandran plots indicate that 87.7% of nonglycine and nonproline residues fall in the most favored regions and 10.9% in the allowed regions. Electron density maps failed to indicate an unambiguous position of one loop, Glu123–Asn127 (Figs 2 and 3), at the surface of the protein. Moreover, the two first N-terminal residues (Ala5 and Thr6) were not detected in the density map.

Figure 3 shows the 3D structure of the psychrophilic TAE4 enzyme. The molecular architecture follows the pattern of the known class C β -lactamases structures, with an all- α domain and an α/β domain with the active site Ser64 located in a depression between the two domains at the N-terminus of the $\alpha 2$ hydrophobic helix. The disordered and unobserved loop is located at one edge of the active site.

The enzymes from *Psy. immobilis* A5 and *S. marcescens* were modeled from either *Citrobacter freundii*

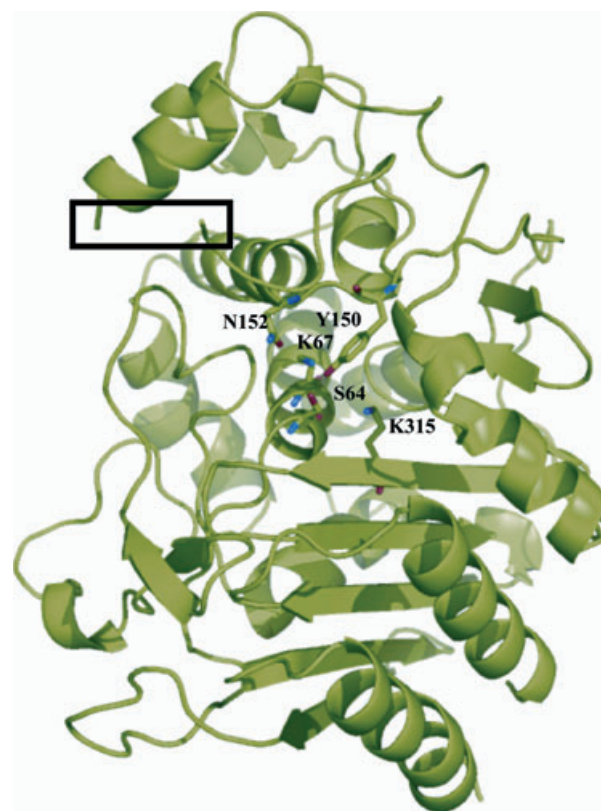


Fig. 3. Crystal structure of the class C β -lactamase TAE4 from *Pse. fluorescens*. The important residues of the active site are labeled. The unobserved loop is indicated by the black box.

Table 4. Data collection and refinement statistics for *P. fluorescens* β -lactamase TAE4. Values listed in parentheses are for the highest resolution (2.3–2.2 Å). $R_{\text{factor}} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$. R_{free} was calculated with 5% of the reflections set aside randomly throughout the refinement.

Data collection	
Wavelength (Å)	0.9797
Resolution (Å)	10–2.2
Space group	P2 ₁
Unit cell parameters	$a = 43.6$ Å, $b = 69.7$ Å, $c = 53.9$ Å, $\beta = 90.9^\circ$
Number of observed reflections	57 181 (7667)
Unique reflections	15 724 (2038)
Completeness (%)	95.4 (99.9)
R_{merge} (%)	3.8 (6.1)
Average $I/\sigma I$	23.94 (16.62)
Refinement statistics	
Resolution range	99–2.2
$R_{\text{factor}}/R_{\text{free}}$ (%)	17.2/25.4
Average B -value for whole chain (Å ²)	34.90
2662 protein atoms	34.53
67 solvent atoms	35.66
rmsd from ideality	
Bonds (Å)/angles (°)	0.005/0.020
Ramachandran plot	
Most favored, additional, generously allowed (%)	89.3/10.0/0.0

(Protein Data Bank code 1FR1, 2.0 Å) or *E. cloacae* P99 (Protein Data Bank code 1XX2, 1.88 Å). They share 35.6% and 43.1% sequence identities, respectively, with their templates, and the obtained models are reliable as indicated by the Ramachandran plot (data not shown). These different model structures and the crystal structures of the psychrophilic enzyme TAE4 from *Pse. fluorescens* (Protein Data Bank code 2QZ6, 2.2 Å) and the mesophilic homolog from *E. cloacae* 908R (Protein Data Bank code 1Y54, 2.1 Å) were compared in order to identify the interactions and the structural features potentially involved in the low stability and structural flexibility of the psychrophilic enzymes.

The overall folding is identical for all the enzymes. Superimposition of the four proteins shows quite low rmsd values (Table 3). Moreover, the conformations of the catalytic triad and the specificity pocket are very similar. Only subtle modifications of the enzyme conformation therefore account for the low stability of cold enzymes. In this context, the disordered region, not observed in the crystal structure of the 908R enzyme and close to the catalytic pocket, is assumed to

be partly responsible for the larger flexibility of the cold β -lactamase and for its ability to hydrolyze large substrates.

Both psychrophilic β -lactamases have fewer ion pairs (3) than the mesophilic ones (5 and 11), showing that electrostatic effects may play a role in the stability of the latter proteins (Table 3). One salt bridge (Glu272–Arg148) involving residues close to the active site is conserved among the four enzymes and probably contributes to the activity. The ion pairs present in the mesophilic but absent in the psychrophilic enzymes are distributed throughout the whole structure. In addition, the number of hydrogen bonds between side chains is also slightly smaller in the case of cold β -lactamases. Even though the mesophilic enzymes have more hydrophobic residues, the hydrophobic contacts and aromatic interactions are similar in all enzymes.

Frequently, alterations of the accessible surface of nonpolar side chains and of the accessible charged surfaces are observed in cold-adapted enzymes. Polar and apolar accessible surface areas were therefore also calculated, but they seem to be not correlated with thermal stability in the present cases.

Discussion

The determination of the crystal structure of a psychrophilic class C β -lactamase, from *Pse. fluorescens* TAE4, and its comparison with one psychrophilic and two mesophilic homologs, allowed a detailed structural analysis to obtain insights into features involved in cold adaptation. Although the four proteins have a very similar fold that is characteristic of class C β -lactamases, subtle sequence and structure differences could be seen.

No significant differences in the number and nature of residues were observed around the active site (within 12 Å from the catalytic serine) of the four proteins. However, one loop at one edge of the active site (Glu123–Asn127) of the *Pse. fluorescens* β -lactamase was undetectable in the electron density map and was therefore assumed to be disordered, which is not the case for the mesophilic homolog 908R. This flexibility, in spite of the steric hindrance of the substrate, is thought to be partially responsible for its unexpected activity on large penicillin substrates. The kinetic parameters of the *Pse. fluorescens* β -lactamase unambiguously show that the psychrophilic enzyme is more active on large substrates (26–130 times), although the active site structure and composition are identical to those of mesophilic β -lactamases. This indicates that the active site of the *Pse. fluorescens* β -lactamase is more easily accessible to large substrates and should

be more dynamic in solution, i.e. flexible in a broad sense. Furthermore, the higher K_m (lower apparent affinity) also suggests a more mobile active site that binds the substrates weakly. In addition, the more flexible conformation of *Pse. fluorescens* β -lactamase would allow easier access of the water molecule in the active site of the enzyme, accelerating the deacylation. These assumptions would also explain the low thermal and chemical stability of the enzyme. It should be noted that these results parallel those obtained for a psychrophilic α -amylase, the latter showing higher activity on large and branched polysaccharides, with, however, a higher K_m , when compared with a mesophilic homolog [33].

Moreover, electrostatics seems to play a major role in the cold adaptation of the present β -lactamases. Indeed, the cold enzymes have a lower total number of ionic interactions than the mesophilic ones. Even though the differences may not appear to be dramatic (only two between the *S. marcescens* enzyme and the cold enzymes), it has already been shown that a single ion pair difference can reflect adaptation to low or high temperatures [23,34]. A strong correlation was also found between thermal stability and the content of basic residues. The psychrophilic enzymes have a slightly lower arginine content and a higher lysine content than their mesophilic homologs, a characteristic of several cold-adapted enzymes [35]. Arginine is a stabilizing residue [36] because of the ability of its guanidinium group to form five hydrogen bonds with surrounding residues, as well as two salt bridges with acidic groups. In addition, lysine residues are more flexible than arginine. Finally, it was also observed that both psychrophilic β -lactamases are overall negatively charged, in contrast to their mesophilic homologs, which supports the conclusion that charges and electrostatics are probably involved in the temperature adaptation.

Other differences were also observed. Given the mean errors of 0.15 Å on coordinates (Luzzati plot), the psychrophilic β -lactamases are characterized by a decreased number of hydrogen bonds, possibly rendering the structure more flexible. To confirm this tendency, higher-resolution structures would be necessary to improve the accuracy of those geometries. In addition, even though the number of hydrophobic contacts is not correlated with the thermostability, the number of hydrophobic aliphatic residues, such as alanine, valine, leucine, and isoleucine, is smaller for the cold β -lactamases. Several examples show that hydrophobicity is positively correlated with the thermostability [37–39].

The number of prolines is also slightly lower for both psychrophilic β -lactamases. Prolyl residues can

adopt only a few conformations and restrict the available dihedral angles of the preceding residue; thus, proline has the lowest conformational entropy and contributes to the local rigidity of the peptidic backbone.

Previous crystallographic studies have indicated that, in addition to the features already mentioned, some cold-adapted enzymes may be characterized by a decreased number of disulfide bonds, an increased number of glycine residues, a reduced apolar fraction in the core, higher accessibility of the active site, and increased exposure of apolar residues to the solvent, as compared with their mesophilic and thermophilic counterparts [40,41]. The present cold β -lactamase enzymes do not seem to use these strategies for cold adaptation.

In conclusion, the crystal structure of the psychrophilic class C β -lactamase from *Pse. fluorescens* TAE4 provides additional insights into cold adaptation of enzymes. Of all the structural features analyzed, those that may contribute to the intramolecular flexibility of TAE4 and its cold homolog from *Psy. immobilis* are a lower content of prolines, decreased numbers of ion pairs and hydrogen bonds, a lower percentage of arginine in comparison with lysine, and a lower number of hydrophobic aliphatic residues.

Experimental procedures

Amino acid sequence determination

The primary structure of the protein was determined by N-terminal Edman degradation and sequence analysis of overlapping peptides generated by digesting separate samples of the protein with Lys-C proteinase, N-Asp protease, and Arg-C protease. The correctness of the sequence of each peptide was controlled by mass analysis using a Tofspec SE MALDI-TOF analyzer (Micromass, Wythenshawe, UK). Edman degradation was carried out using a 476 protein sequenator (Applied Biosystems, Foster City, CA, USA). The molecular mass of the native protein was determined by ESI MS on a Q-TOF mass analyser (Micromass).

Production and purification of *Pse. fluorescens* TAE4

Production and purification of the *Pse. fluorescens* TAE4 β -lactamase are described elsewhere [2].

Kinetic parameters

Kinetic parameters were determined at 30 °C in 50 mM sodium phosphate buffer (pH 7.0), on the basis of either

complete time-courses [42] or initial rates. Low K_m values were derived from substrate competition experiments [43]. The temperature of 30 °C was selected because most previous data published for β -lactamases, obtained using the same substrates and techniques, have been recorded at this temperature. The range of cephalosporin substrate concentrations used in the k_{cat} and K_m determinations were 100, 150 and 15–200 μ M for nitrocefin, cefazolin, and cephalixin, respectively. The penicillin substrate concentration used in the k_{cat} determination was 1000 μ M for all substrates. The concentrations of benzylpenicillin, ampicillin, carbenicillin, oxacillin and cloxacillin used in the K_m determination were 2–8, 1–40, 1–100, 0.1–3 and 0.005–0.04 μ M, respectively.

Stability and thermal and urea denaturation

Kinetic stability parameters were determined from fluorescence quenching (281 and 343 nm for excitation and emission, respectively) of the enzymes (20 μ g·mL⁻¹) at various temperatures. The buffers used were 10 mM Hepes and 0.2 M NaCl (pH 8.2) for *S. marcescens* and *E. cloacae* 908R, and 50 mM NaCl/P_i (pH 7.0) for *Pse. fluorescens* TAE4 and *Psy. immobilis* A5. The denaturation rate constants, k_d , were determined by measuring the fluorescence intensity as a function of time, following the equation:

$$F_t = [(F_0 - F_\infty)\exp(-k_d t)] + F_\infty$$

where F_0 , F_t and F_∞ are the fluorescence intensities at $t = 0$, $t = t$ and $t = \infty$, respectively.

Thermodynamic parameters were determined from fluorescence quenching of the psychrophile enzyme TAE4 (20 μ g·mL⁻¹) at various urea concentrations. The buffers used were the same as for the thermal denaturation experiments. Different thermodynamic parameters can be deduced from the experimental curves. As described by Vanhove *et al.* [44], the free energy difference between the native (N) and the denatured (D) form without denaturing agent, ΔG_{D-N}^0 , is calculated by adjusting the observed fluorescence (F_{obs}) as a function of the urea concentration:

$$F_{obs} = \frac{F_N + F_D \cdot \exp(a)}{1 + \exp(a)}$$

with

$$F_N = F_N^0 + p \cdot [\text{urea}]$$

$$F_D = F_D^0 + q \cdot [\text{urea}]$$

where F_N and F_D are the intrinsic fluorescence of the native and denatured form, respectively, and p , q are parameters taking into account the observed linear dependence of the

intrinsic fluorescence of the native and denatured form as a function of urea concentration.

$$a = \frac{-\Delta G_{D-N}^0 + m_{D-N} \cdot [\text{urea}]}{RT}$$

where m_{D-N} is the slope of the line relating the free energy difference between the native (N) and denatured (D) form at a given urea concentration to the urea concentration. The denaturant concentration necessary to have a ratio N/D = 1 is obtained from:

$$C_m = \frac{\Delta G_{D-N}^0}{-m_{D-N}}$$

Crystallization and data collection for *Pse. fluorescens* TAE4 β -lactamase

Crystals of the cold *Pse. fluorescens* β -lactamase were grown at 4 °C by the hanging drop vapor diffusion method, by mixing 4 μ L of a 10 mg·mL⁻¹ protein solution with the crystallization solution containing 20% poly(ethylene glycol) 6 K in 0.1 M Tris (pH 8.0). Crystals were flash-frozen in a cold liquid nitrogen stream (100 K) with 35% glycerol as cryoprotectant. Diffraction data were collected at beam line BM30A (European Synchrotron Radiation Facility, Grenoble, France) on a MarResearch CCD.

Data processing, molecular replacement and refinement of *Pse. fluorescens* TAE4 β -lactamase

Data were processed with the HKL suite package [45]. A molecular replacement solution was found using AMORE [46] with the molecular model of the class C β -lactamase from *E. cloacae* P99 (Protein Data Bank entry 2BLT) [47]. Refinement was performed with the SHELXL97 program [48]. Electron density maps were inspected with the graphic program XTALVIEW [49], and the quality of the model was analyzed with the program PROCHECK [50].

Homology modeling

Sequence analysis was performed using CLUSTALW. *C. freundii* (Protein Data Bank code 1FR1) and *E. cloacae* P99 (Protein Data Bank code 1XX2) were selected as the most appropriate templates for *Psy. immobilis* and *S. marcescens*, respectively. Both amino acid sequences were aligned by means of the ESYMPRED3D program [51]. This automated homology modeling program compares results from various multiple alignment algorithms to derive a 'consensus' alignment between the target sequence and the template sequence. Furthermore, a 3D model (built with MODELER) was also provided with ESYMPRED3D. Structure quality verification of the model was performed with PROCHECK 3.0.

Structure analysis

The STING Millennium Suite, which is web-based software, was used to analyze the structures of the four proteins [52]. Two oppositely charged residues were identified as an ion pair if their atoms were within 2.0–4.0 Å. Hydrogen bonds and hydrophobic contacts were defined within 2.0–3.2 Å and 2.0–3.8 Å, respectively. Total, polar and apolar surface-accessible areas were calculated with NACCESS using a probe radius of 1.4 Å [53].

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