Hyperstability and Substrate Promiscuity in Laboratory Resurrections of Precambrian β-Lactamases

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S *Supporting Information*

ABSTRACT: We report a sequence reconstruction analysis targeting several Precambrian nodes in the evolution of class-A β-lactamases and the preparation and experimental characterization of their encoded proteins. Despite extensive sequence differences with the modern enzymes (∼100 amino acid differences), the proteins resurrected in the laboratory properly fold into the canonical lactamase structure. The encoded proteins from 2–3 billion years (Gyr)-old β-lactamase sequences undergo cooperative two-state thermal denaturation and display very large denaturation temperature enhancements (∼35 °C) relative to modern β-lactamases. They degrade different antibiotics in vitro with catalytic efficiencies comparable to that of an average modern enzyme. This enhanced substrate promiscuity is not accompanied by significant changes in the active-site region as seen in static X-ray structures, suggesting a plausible role for dynamics in the evolution of function in these proteins. Laboratory resurrections of 2−3 Gyr-old β-lactamases also endowed modern microorganisms with significant levels of resistance toward a variety of antibiotics, opening up the possibility of performing laboratory replays of the molecular tape of lactamase evolution. Overall, these results support the notions that Precambrian life was thermophilic and that proteins can evolve from substrate-promiscuous generalists into specialists during the course of natural evolution. They also highlight the biotechnological potential of laboratory resurrection of Precambrian proteins, as both high stability and enhanced promiscuity (likely contributors to high evolvability) are advantageous features in protein scaffolds for molecular design and laboratory evolution.

Antibiotic resistance genes are widespread in natural environments, partly as a result of human activities such as the use of antibiotics in agriculture and fish farming.[1] In all likelihood, however, antibiotic resistance existed before the modern use of antibiotics. In fact, resistance genes have been found in unpolluted sites such as remote Alaskan soil,[2a] the bottom of the Pacific Ocean,[2b] and even 30 000-year-old permafrost sediments.[2c] Furthermore, β-lactamases, the enzymes responsible for the primary mechanism of resistance toward β-lactam antibiotics, are widely distributed throughout the bacterial domain of life and are believed to have originated billions of years (Gyr) ago.[3] The role antibiotics and antibiotic resistance play in modern natural environments is not fully understood,[1] so one can only speculate about the reasons for the existence of β-lactamases that are several billion years old. It has been suggested that they originated as a mechanism of defense to stave off competitors[3a] or had a role in signaling.[4]

Laboratory resurrection of ancestral proteins[5] has been shown to provide insight into the ancient properties of biomolecules as well as the intracellular and extracellular environments hosting these proteins. Here we report the derivation of statistically probable sequences for several Precambrian β-lactamases, the preparation in the laboratory of the corresponding proteins, and their experimental characterization in terms of stability, function, and structure.

We used a set of chromosomal sequences of extant class-A β-lactamases [Tables S1 and S2 and Figure S1 in the Supporting Information (SI)] to construct a phylogenetic tree encompassing Gram-positive and Gram-negative bacteria (Figures S2 and S3). The sequences were derived from a search in the NCBI database of complete genomes, and thus, plasmidic lactamases were not included. To avoid potential complications during the sequence reconstruction process that could arise from recent evolution during the antibiotic era, we determined that none of the sequences came from clinical isolates. The 75 sequences selected provide uniform coverage of the phyla in Bacteria, including the Proteobacteria, Actinobacteria, Firmicutes, and Cytophaga—Flexibacter—Bacteroides (CFB) lineages, and the topology of the inferred phylogenetic tree is sufficiently close to an accepted phylogeny of these organisms[6] (Figure S4) to allow us to target well-defined Precambrian nodes (for details, see Materials and Methods in the SI). We focused our sequence reconstructions of β-lactamases on the last common ancestors (CAs) of enterobacteria (ENCA), Gammaproteobacteria (GPBCA), various Gram-negative bacteria (GNCA), and various Gram-positive and Gram-negative bacteria (PNCA). Estimates of divergence times[6]

indicate that these ancestors inhabited Earth about 1, 1.5, 2, and 3 Gyr ago, respectively. We used Bayesian statistics to reconstruct the sequences of the β-lactamases corresponding to these Precambrian nodes and accounted for the protein corresponding to the most probabilistic sequence (i.e., the sequence with the most probable amino acid at each position) for each phylogenetic node of interest (Figure S5). The reconstructed proteins have substantial amino acid sequence differences relative to extant β-lactamases and also to a consensus sequence computed from extant β-lactamases (Table S3). The phenotypic robustness of the laboratory resurrection was supported by measurements on additional sequences at the GNCA node derived from a Monte Carlo sampling of the posterior probability distribution (Figures S6−S10 and Table S4).

The laboratory resurrections of the ancestral β-lactamases were characterized to determine their structural, physicochemical, and functional properties (see Materials and Methods). Crystallization and 3D structure determination of the lactamases corresponding to the GNCA and ENCA nodes showed that they share the extant lactamase fold despite extensive sequence differences (122 and 55 amino acid differences, respectively, relative to TEM-1 β-lactamase[7] (Figure 1). Closer inspection revealed that no important conformational changes reside in the

ancestral GNCA and ENCA structures relative to the TEM-1 lactamase structure [root-mean-square deviations (RMSDs) of 1.44 and 0.63 Å, respectively] (Figures 1 and S16). Minor movements having RMSD ≥ 2 Å appear in the α+β domain of the GNCA lactamase, corresponding to solvent-exposed loops and the terminal helix, but such movements can be expected. No substantial differences were found in the α domain, and all of the active-site amino acids occupy canonical space (RMSDs of 0.42 and 0.40 Å for GNCA and ENCA, respectively).

The thermal stability of the resurrected Precambrian β-lactamases was studied by differential scanning calorimetry (DSC). The resurrected 2−3 Gyr-old β-lactamases were found to be highly stable, as revealed by their denaturation temperatures (T_m), which were ∼35 °C higher than those of extant lactamases, including that from the thermophilic *Bacillus licheniformis* (Figures 2A and S14). Also, the effect of scan rate
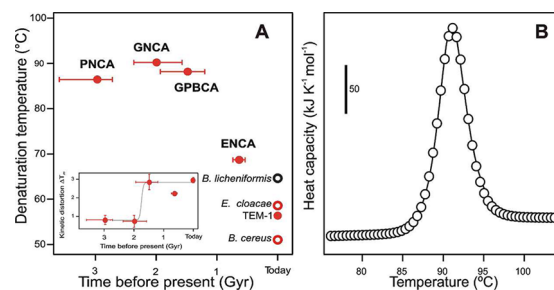
Figure 2. (A) Plot of denaturation temperature vs geological time for β-lactamases, with uncertainties in geological time from ref 6 (see Figure S14 for details). Inset: kinetic distortions in the thermal denaturation of β-lactamases as measured by the effect of scan rate on the DSC profiles (see Figure S15 for details). (B) DSC profile for thermal denaturation of the resurrected GNCA β-lactamase: ○, experimental data; solid line, best fit using the two-state equilibrium model (see Figure S15 for details and additional fits).
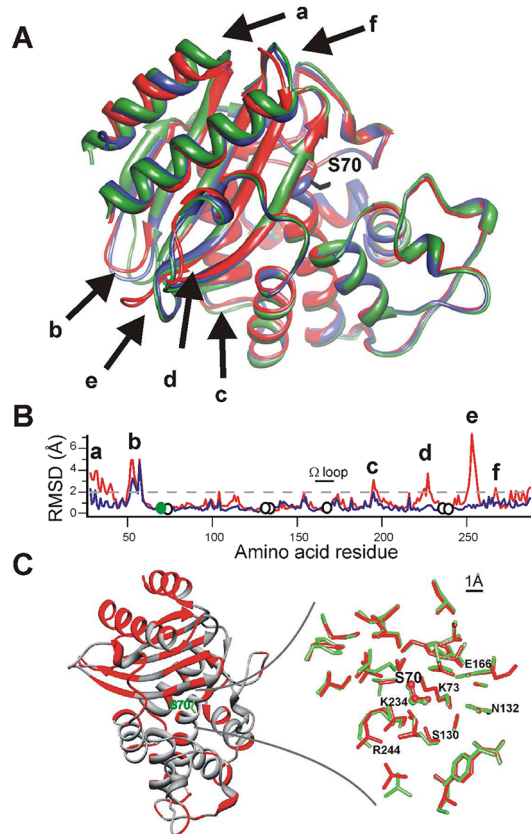

Figure 1. (A) Structural comparison of the TEM-1 β-lactamase (PDB entry 1btl; green) and the resurrected GNCA (PDB entry 4b88; red) and ENCA (PDB entry 3zdj; blue) β-lactamases. The catalytic serine S70 is shown. (B) RMSD plot. Circles represent the active-site positions (green for S70). Minor structural differences (labeled a−f) are seen only in the α+β domain. (C) (left) GNCA lactamase structure. Positions with differences relative to modern TEM-1 β-lactamase are shown in red. (right) Side-chain conformations within a sphere of 8 Å centered at S70 for GNCA (red) and TEM-1 (green) β-lactamases. Active-site residues are labeled.

on the DSC profiles indicated that kinetic distortions are substantially smaller for the resurrected ancestral lactamases (Figure 2A inset and Figure S15), suggesting enhanced kinetic stabilization against irreversible denaturation processes.[8] In fact, the DSC profiles for the resurrections of 2−3 Gyr-old β-lactamases are well-described by a simple native-unfolded, two-state equilibrium model (Figures 2B and S15).

It is unlikely that the enhanced stability shown in Figure 2A was obtained by chance or through biases associated with the sequence reconstruction process because the sequence differences are extensive (∼100 amino acid replacements) and, as is well-known, most mutations in a protein are destabilizing.[9,10] Moreover, the T_m enhancement of ∼35 °C is substantially larger than those reported in stability engineering studies of modern β-lactamases. For instance, combinatorial recruitment of consensus mutations led to a T_m enhancement of 9 °C for the β-lactamase of *Enterobacter cloacae*,[11] and consensus variants of TEM-1 β-lactamase obtained through intense neutral drifts displayed T_m enhancements of up to 7 °C.[12] In fact, the highest T_m reported to date for a variant of TEM-1 β-lactamase is 69 °C,[13] which is still ∼20 °C below the T_m values for the resurrected 2−3 Gyr-old β-lactamases (Figure 2A).[14] It is also noteworthy that two other recent Precambrian protein resurrection studies[5d,e] involving thioredoxins and elongation factors also found T_m enhancements of 30−35 °C upon "traveling back in time" 2−3 Gyr. The fact that thioredoxins, elongation factors, and β-lactamases differ in structure, size, and function strongly suggests that hyperstability

should be a general outcome for many Precambrian resurrection efforts, likely reflecting the adaptation of Precambrian proteins to the hot conditions of the ancestral oceans.

In vitro measurements of the catalytic properties for the hydrolysis of several antibiotics (Figure S11) revealed that the resurrections of 2−3 Gyr-old β-lactamases have somewhat decreased values of k_{cat}/K_m for penicillin antibiotics but substantially enhanced values for third-generation antibiotics (Figures 3A and S12 and Table S5). Actually, while the extant
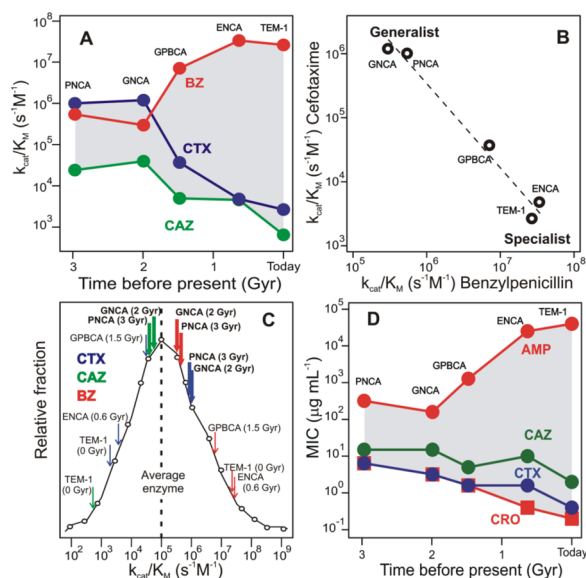

Figure 3. (A) k_{cat}/K_m for the in vitro hydrolysis of antibiotics catalyzed by extant and resurrected β-lactamases. (B) Generalist-to-specialist conversion as illustrated by the catalytic efficiencies for benzylpenicillin (BZ) and cefotaxime (CTX). (C) Distribution of catalytic efficiencies. Open circles represent values for modern enzymes from ref 15, and arrows indicate values for the β-lactamases studied in this work. Thicker and longer arrows are used for the substrate-promiscuous laboratory resurrections of the 2−3 Gyr-old β-lactamases. (D) MICs determined using an *E. coli* strain transformed with plasmids containing the resurrected β-lactamases. In (A) and (D), shading is meant to illustrate the change in the range of values with geological time. The uncertainties in geological time are given in Figure 1A. The Michaelis−Menten parameters and MICs (together with the associated errors) are collected in Tables S5 and S6. BZ, ampicillin (AMP), and the third-generation antibiotics CTX, ceftazidime (CAZ), and ceftriaxone (CRO) (Figure S11) were studied.

TEM-1 β-lactamase is clearly a penicillin specialist, the GNCA and PNCA β-lactamases show roughly similar levels of catalytic efficiency for the different antibiotics studied (penicillin and third-generation) and can be regarded as substrate-promiscuous enzymes (Figure 3B). Bar-Even et al.[15] recently analyzed the catalytic efficiencies for several thousand (extant) enzymes and found that the "average enzyme" has an moderate efficiency of $k_{cat}/K_m \sim 10^5$ M^{-1} s^{-1}, which is much lower than the diffusion limit of 10^8−10^9 M^{-1} s^{-1}. Figure 3C shows the distribution of catalytic efficiency for extant enzymes reported in ref 15 with some added arrows that locate the catalytic efficiency levels of the β-lactamases studied here (see Figure S13 for the K_m and k_{cat} distributions). The efficiencies of the GNCA and PNCA β-lactamases toward the different antibiotics used are similar to that of the average extant enzyme. The resurrected 2−3 Gyr-old β-lactamases can thus be described as moderately efficient promiscuous enzymes. Broad substrate scope in lactamases

from clinical isolates has sometimes been linked to significant structural alterations.[16] Thus, it is surprising that the structures determined for the promiscuous GNCA β-lactamase and the modern TEM-1 penicillin specialist did not show significant differences in the active-site region (Figure 1C) or the critical Ω loop (Figure 1B). While subtle structural effects could certainly play a role,[17] it appears plausible that the evolution of function in this system is related to dynamic effects[18,19] that may not be apparent in the static X-ray structures.[20,21]

To assess the capability of each resurrected ancestral β-lactamase to confer antibiotic resistance within a modern organism, we transformed a DH5α *Escherichia coli* strain with a PBR322 plasmid containing a reconstructed gene sequence encoding a resurrected protein. To minimize alterations in protein export to the periplasm, the reconstructed ancestral sequence was fused to the 23-residue peptide signal sequence from the extant *E. coli* lactamase. Minimum inhibitory concentrations (MICs) were subsequently determined for the penicillin-family antibiotic ampicillin and also for several third-generation antibiotics (Figure 3D and Table S6). The resurrected β-lactamases showed decreased MICs for ampicillin (compared with the extant β-lactamase from *E. coli*) but enhanced MICs for other antibiotics, in qualitative agreement with the in vitro catalytic efficiencies described above (compare panels A and D in Figure 3).[22] The capability of the resurrected Precambrian β-lactamases to confer antibiotic resistance within a modern organism opens up the interesting possibility of performing directed laboratory evolution of these proteins in a straightforward manner (i.e., through in vivo selection for enhanced resistance).

Several published studies of laboratory resurrection of ancestral proteins have provided narratives describing evolutionary adaptations over planetary time scales. One plausible narrative suggested by the present results is that the enhanced stability of our resurrected β-lactamases reflects the adaptation to high temperatures predicted to be associated with Earth's early oceans that hosted life.[5d,e] Consequently, the decrease in stability over several Gyr (Figure 2) may reflect the cooling of the oceans, although the existence of stability/activity trade-offs cannot be ruled out. The resurrected proteins' enzymatic promiscuity may be related to the wide variety of substances Precambrian lactamases had to hydrolyze[3a] or may simply reflect generalist properties of the first stages in the evolution of lactamase enzymes.[23] This generalist property is revealed by the capability of our resurrections to hydrolyze different types of antibiotics, including modern third-generation ones (Figure 3A,D). Their functional conversion from generalists to penicillin specialists over a several-Gyr time scale (Figure 3) could be an adaptation to the development of efficient penicillin-like antibiotics by other organisms (interestingly, the divergence time of fungi is ~1.2 Gyr before present).[6] The overall enzymatic and physicochemical characterization of the resurrected β-lactamases thus supports the notions that Precambrian life was thermophilic and that proteins can evolve from generalists (displaying substrate promiscuity) to specialists capable of efficient enzymatic turnover during the course of natural evolution.

The availability of resurrected Precambrian β-lactamases opens up new possibilities in the study of the emergence of antibiotic resistance. For instance, it should now be possible to perform laboratory replays of the molecular tape of lactamase evolution to probe the molecular determinants of the efficiency of lactamases in adapting to different types of antibiotics. From a different perspective, we point out that both high stability and

promiscuity (likely contributors to high evolvability) are advantageous features in protein scaffolds for molecular design and laboratory evolution. High stability allows destabilizing but functionally useful mutations to be accepted without compromising proper folding.[24a] Substantial promiscuity is essential for laboratory evolution of high levels of a targeted function.[24b] The reported simultaneous occurrence of both features thus supports the potential of laboratory resurrection of Precambrian proteins in protein engineering and protein biotechnology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org. Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank as entries 4b88 and 3zdj.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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(14) Also, a T_m enhancement of ~35 °C is larger than those typically achieved by rational design of protein stability. For instance, a 10 °C enhancement for a model enzyme using RosettaDesign was reported. See: (a) Korkegian, A.; Black, M. E.; Baker, D.; Stoddard, B. L. *Science* **2005**, *308*, 857. Recently, a variant of the small protein RNase Sa (98 residues vs the ~260 residues of β-lactamases) was successfully prepared with a 28 °C higher T_m than the wild-type protein. See: (b) Fu, H.; Grimsley, G.; Scholtz, J. M.; Pace, C. N. *Protein Sci.* **2010**, *19*, 1044. However, this large stabilization resulted from a combination of several stabilizing mutations previously identified using different rational approaches over a period of ~10 years, which actually emphasizes the difficulty of achieving T_m enhancements of several tens of degrees by rational design.

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(17) For instance, the mutation E104K is often found in clinical isolates, while E104 in TEM-1 β-lactamase is changed (to D, not K) in the highly promiscuous GNCA and PNCA lactamases. However, it does not appear likely that the E104D mutation by itself is responsible for the broad substrate scope, as there is also a D at position 104 in the GPBCA lactamase, which is much less promiscuous (Figure 3B).

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(21) Indeed, given the significant difference in the sizes of the antibiotics studied (Figure S11), increased flexibility in functionally relevant regions of the promiscuous GNCA structure would seem plausible. Preliminary computational and experimental analyses (work in progress) point to enhanced flexibility in the catalytic α domain and enhanced rigidity in the $\alpha+\beta$ domain, a scenario that could rationalize the simultaneous occurrence of hyperstability and extended substrate scope. We note that the relation between conformational rigidity and stability, although plausible, does not necessarily hold in all cases. See: Jaenicke, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2962.

(22) The correlation between MIC and catalytic efficiency is not perfect (e.g., the increase in MIC for CTX upon "traveling back in time" 2−3 Gyr is smaller than the corresponding increase in catalytic efficiency), possibly reflecting molecular requirements related to export to the periplasm and subsequent folding of the lactamase enzymes. See: Chatzi, K. E.; Sardis, M. F.; Karamanou, S.; Economou, A. *Biochem. J.* **2013**, *449*, 25. For instance, export through the bacterial Sec system likely requires the preproteins to remain partially unfolded prior to interaction with the Sec channel, and this partial unfolding could be hindered by high stability.

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