Supporting information

Hyperstability and substrate promiscuity in laboratory resurrections of Precambrian β-lactamases

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MATERIALS AND METHODS

Phylogenetic analysis and ancestral sequence reconstruction

Chromosomal sequences of extant β-lactamases (Tables S1 and S2) were retrieved from the **October**-2011 release of the complete genomes database available at NCBI (www.ncbi.nlm.nih.gov/genome) and, therefore, plasmidic sequences were not included. To further avoid potential complications during the reconstruction process that may arise from recent evolution during the antibiotic era, we determined that none of the sequences came from clinical isolates. Sequences were aligned using MUSCLE (Figure S1). The phylogeny of the Class A β-lactamases was constructed by using the Bayesian method[1] as implemented in the program MrBayes 3.1.2. This analysis used two independent Markov-chain Monte Carlo runs, with four chains each, and was performed for 4,000,000 generations to ensure convergence. Trees were sampled every 1000 generations for each run resulting in a total of 8000 trees, from which a consensus tree was obtained with high probabilities at the nodes (Figure S2 and S3).

Horizontal gene transfer (HGT) is known to be responsible for the spread of antiobiotic resistance determinants and the rate at which resistance genes are mobilized from chromosomes to plasmids has been reported to have increased during the last 60 years[2]. Remarkably, the consensus tree derived from the set of chromosomal sequences (Figures S2 and S3) does not reveal large HGT distortions. First of all, the Bayesian analysis places most sequences of extant lactamases in their "correct" phylogenetic groups (the groups to which the corresponding species belong). Secondly, the overall topology of the consensus tree is reasonably close, not only to the lactamase tree published by Hall and Barlow (ref. 3a in the main text), but also with the Eubacteria tree reported in the Time Tree of Life (ref. 12 in the main text). To make this latter point clear, we include in figure S4 a schematic representation of the Eubacteria tree which can be compared with our consensus lactamase tree of Figures S2 and S3. The only clear evidence of HGT appears to be the presence of a few sequences from Gram negative bacteria that cluster with Terrabacteria group, but this result is in agreement with a previous report (ref. 3a in the main text). There is also some difference in the attachment of the CFB group, although this is mainly related to the fact that we have followed Hall and Barlow (ref. 3a in the main text) and used the CFB group as an outgroup.

The above differences notwithstanding, the general agreement between the topology of the inferred phylogenetic with an accepted phylogeny of the organisms allows us to target well-defined Precambrian nodes: specifically those corresponding to the last common ancestor of enterobacteria (ENCA), the last common ancestor of gamma-proteobacteria (GPBCA), the last common ancestor of various Gram negative bacteria (GNCA) and the last common ancestor of various Gram positive and Gram negative bacteria (PNCA). These nodes are defined in terms of the Eubacteria tree taken from the Time Tree of Life (see Figure S4) and, therefore, can be dated using the divergence times provided in the Time Tree of Life (ref. 6 in the main text), but they can also be easily identified in our inferred consensus tree for lactamases (Figures S2 and S3), opening up the possibility ot targeting the sequence reconstruction exercise to these Precambrian nodes. It is also important that the Bayesian,

Markov-chain Monte Carlo analysis assigns high posterior probability values to the targeted nodes (PP≥0.9, see Figure S2). This result is relevant because it has been shown that sequence reconstructions at nodes with high posterior probability are robust to uncertainties about the underlying tree[3]. Finally, it must be noted that the reported experimental stability determinations with the resurrected proteins (Figure 2) are fully consistent with the claim that our sequence reconstruction process targets Precambrian nodes. Thus, we find a rate of increase in denaturation temperature of about 10 degrees per billion years, which agrees with the the rates found in laboratory resurrection of Precambrian elongation factors (ref. 5d in the main text) and thiroedoxins (ref. 5e in the main text), and also with the rate of change of the temperature of the oceans in the Precambrian as inferred from the isotopic composition of rocks (ref. 5d in the main text).

Ancestral sequences were reconstructed using PAML[4] version 4.4e with the Wag substitution model and incorporating the gamma distribution for variable replacement rates across sites. β-lactamases are rather homogeneous in length and only a few sequence insertions were detected and, furthermore each one of these insertions occurred in a very small number of sequences (2-3 sequences and 1 insertion of 1 amino acid apperring in 7 sequences). These insertions were deleted because they are highly unlikely to have appeared in the common ancestors and, in any case, they are poorly determined in the reconstruction process. Removal of those insertions in the extant sequences would lead to lenghts spanning the range 262-267 amino acids for the mature proteins, with most of the sequences actually being 262 amino acids in length. Therefore, the reconstructred sequences were reduced to a common length of 262 amino acid residues. This is very unlikely to have any signficant effect because the few external residues deleted occur at the highly flexible C-terminal end, which is removed from the active-site (in any case, those positions are again poorly determined in the recontruction process). Profiles of probability for the most probabilistic residue versus position are given in Figure S2 for the sequences reconstruted for the ENCA, GPBCA, GNCA and PNCA nodes (Figure S5). These most probabilistic sequences at each node were selected for laboratory resurrection. The reconstructed sequences have substantial amino acid differences with extant β-lactamases and also to a consensus sequence computed from extant ß-lactamases (Table S3). Nevertheless, it is plausible that consensus mutations could make a significant contribution to the enhancement found, but this may only mean that consensus is an approximate/heuristic way to do ancestral sequence reconstruction, as some authors have suggested[5]. It must be noted, nevertheless, that consensus sequence changes have been explored with the TEM-1 lactamase (ref. 12 in the main text) and the Tm enhancements reported are much smaller that those found here.

To test the phenotypic robustness of the laboratory resurrection, alternative protein sequences were also obtained (and subsequently resurrected in the laboratory) by sampling from Monte Carlo runs based on the posterior probability distribution at the GNCA node (Figure S6, S7, S8, S9 and S10, and Table S4).

Protein Expression and Purification

Genes encoding the ancestral β-lactamase enzymes were synthesized and codon-optimized for expression in *E. coli* cells. The genes were cloned into a pET24 vector (Novagen) and transformed in *E. coli* BL21 (DE3) cells (Stratagene, CA). The β-lactamases were purified by osmotic shock followed by gel-filtration chromatography on a Superdex-75 column in 25 mM Hepes, pH 7.0, 150 mM NaCl. Protein purity was verified by SDS-PAGE. In all cases, stock protein solutions were prepared by exhaustive dialysis against 25 mM Hepes buffer, pH 7.0.

Enzyme kinetics

K_m, k_{cat} and k_{cat}/K_m values for the lactamase-catalyzed hydrolysis of the antibiotics (Figure S10) benzylpenicillin (BZ), cefotaxime (CTX) and ceftazidime (CAZ) were determined[6] at 25°C in 50 mM sodium phosphate buffer pH 7.0 (Table S4 an S5). Initial rates were calculated from the change in UV absorbance that ensued from the consumption of no more than 10% of the substrate. k_{cat} and K_m values were calculated by fitting the Michaelis–Menten equation to the profiles of velocity versus substrate concentration (Figures S12 and S13). In a few instances in which K_m was very large, only k_{cat}/K_m values were determined from the linear profiles of velocity versus substrate concentration. In all cases, experiments were done in triplicate.

Antibiotic susceptibility

E. coli DH5-α cells transformed with a pBR322 plasmid encoding TEM-1 or a ancestral β-lactamases were used for antibiotic resistance tests. Minimum inhibitory concentrations (MICs) were determined by broth micro-dilutions[7]. The antibiotics tested (Figure S11) were: cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO) and ampicillin (AMP) (Sigma-Aldrich Inc., St. Louis, MO, EUA). Plates were incubated at 37°C for 18-20 hours and examined for cell growth. The MICs of each transformant, as well as that of the control strain *E. coli* ATCC 25922, were assayed in triplicate with serially diluted (2-fold) antibiotic stocks. In all cases, identical values were obtained and, therefore, the variability between different experiments is less than twofold (Table S6).

DSC experiments

Thermal stabilities of ancestral and modern β-lactamase enzymes (Figures 2, S10, S14 and S15) were measured with a VP-Capillary DSC [Microcal (GE Healthcare) Northhampon, USA] as previously described[8-10]. Protein solutions for calorimetric experiments were exhaustively dialyzed against 25 mM Hepes buffer, pH 7.0 and the buffer from the last dialysis step was used as reference in the calorimetric experiments. Prior to scans with protein solutions, several buffer-buffer baselines were obtained to ensure proper equilibration of the calorimeter. For each protein, experiments at different scan rates (60, 90, 120, 150 and 200 K/h) and at different protein concentrations (within the 0.1-1.5 mg/mL range) were typically performed. Concentrations were determined spectrophotometrically at 280 nm using extinction coefficients and molecular weights calculated from the sequences. No significant concentration effects on thermal denaturation were detected. Small effects of scanning rate were found (Figures 2 and S15) and described in terms of the change in denaturation temperature associated to a ten-fold change in scanning rate (kinetic distortion ΔT_m in inset Figure 2A). These scanning rate effects were

certainly much smaller that the differences in T_m between the extant and resurrected Precambrian β-lactamases.

DSC transitions were calorimetrically irreversible in all cases (i.e., no transitions were detected in re-heating runs performed after stopping the first run at temperature immediately above the calorimetric transtion). This indicattes that irreversible alterations of protein states (likely unfolded or partially unfolded states) take place upon thermal denaturation (ref. 8 in the main text) and may potentially give rise to kinetic distortions in the DSC thermograms, as we have extensively discussed in the literature. However, such distortions are unlikely to be substantial in the case of the GNCA and PNCA lactamases because the scanning rate effect on denaturation temperature (a metric of kinetic distortions) is rather small in these cases (see Inset in Figure 2A and S15). Therefore, analysis of DSC transitions for the GNCA and PNCA lactamases in terms of equilibrium thermodynamics models may be permissible and Figure S15 shows the fit of an equilibrium pseudo-two-state model to these transitions. The model has the general mathematical form of a two-state equilibrium model, but allows for a van`t Hoff enthalpy value (the enthalpy change that determines the temperature dependence of the unfolding equilibrium constant) that may differ from the true unfolding enthalpy (the so-called calorimetric enthalpy). As has been extensively discussed in the field[11], the comparison between the two enthalpy values provides a stringent test of the two-state character of the denaturation process (provided that the low-temperature and high-temperature baselines are well defined in the experimental thermograms, as is the case with the calorimetric profiles reported here). Fits of the pseudo-two-state model to the transitions for the GNCA and PNCA lactamases were excellent and yielded van't Hoff enhtalpy values close to the corresponding calorimetric enthalpy values (Figure S15), strongly supporting the two-state character of the thermal unfolding in these cases. Actually, excellent fits can be obtained using a true two-state model in which only one unfolding enthalpy value (determining both the are under the transition and the temperature-dependence of the equilibrium constant) is used. This latter type of fit is that given in Figure 2B of the main text.

It is to be noted that the analyses described above (using equilibrium thermodynamics models) cannnot be applied to the DSC profiles for GPCA, ENCA and TEM-1 lactamases because, in these cases, the scanning-rate effects are larger (see inset in Figure 2A of the main text and Figure S15) indicating substantial kinetic distortions. In these cases, therefore, the potential deviations from two-state behavior cannot be ascertained from the DSC transitions in a straight forward manner, although it is possible that a more elaborate analysis (along the general lines provided in Sanchez-Ruiz[12]) could lead to a reliable description of the thermal denaturation process (work in progress).

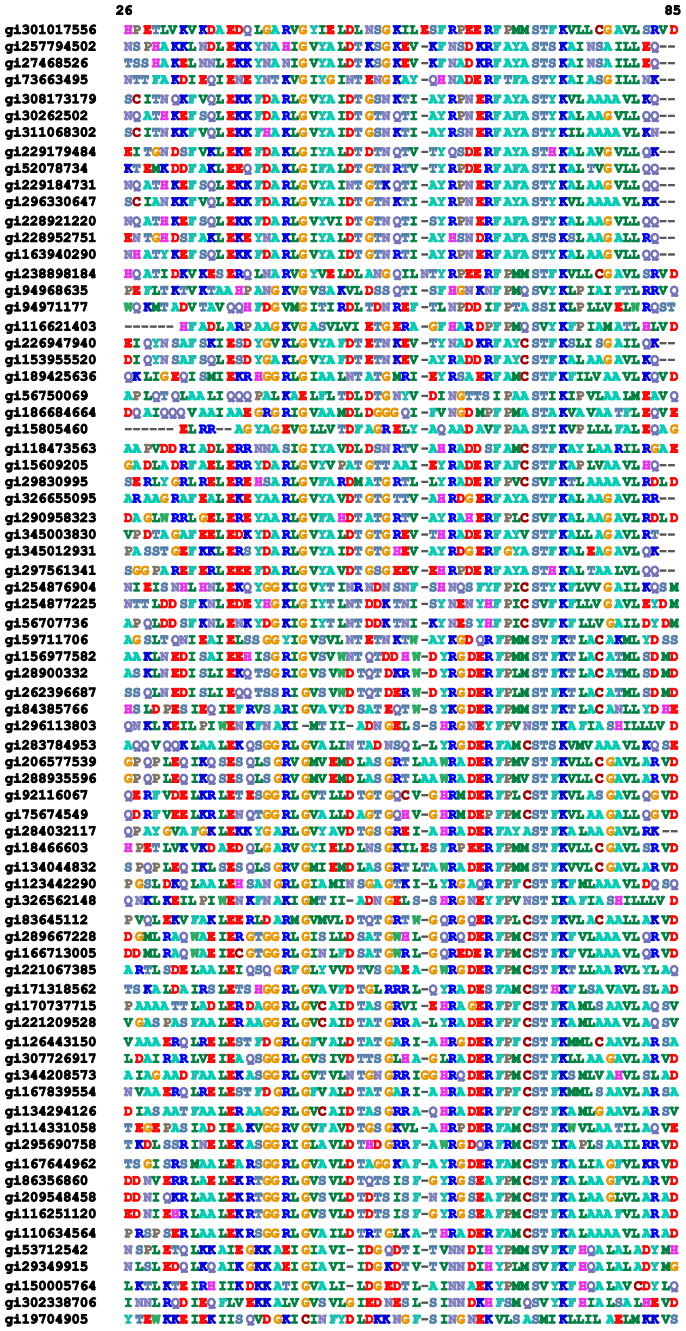
Protein crystallization and structure determination

Crystals of the ENCA and GNCA β-lactamase where grown using the counter-diffusion technique[13]. Good-quality diffracting crystals were obtained at protein concentrations of 30 mg/mL in 25 mM Hepes pH 7.0. Resurrected β-lactamases were crystallized under the following conditions: ENCA: 2.0 M litium sulphate, 0.1M sodium Hepes (pH 7.50) and GNCA: 5.0 M sodium formate, 0.1 M sodium acetate (pH 4.0). The crystal structures

(Figures 1 and S16) were determined by the molecular-replacement method, using as search model the GES-1 β-lactamase structure from *K. Pneumoniae* (PDB.ID 2QPN) solved at 1.1 Å. X-ray crystal structure coordinates have been deposited in the PDB with the following accession codes: ENCA (PDB.ID 3zdj) and GNCA (PDB.ID 4b88). Data collection and refinement statistics are detailed in Table S7.

Although it is often assumed that high stability implies high conformational rigidity, we have not found any clear correlation between B factors and stability. However, although the variation of B-factors along the polypeptide chain can be interpreted in dynamical terms[14], proteins exhibit individual atomic anisotropic motion and collective, large-scale motion over a range of time scales. Dynamics and heterogeneity remains in the crystalline form[15]. Crystal contacts[16] data resolution limit[15], radiation damage, etc., can definitively affect the values determined for the B-factors making difficult to correlate B-factor and stability. We also note that the relation between conformational rigidity and stability, although plausible, does not necessarily hold in all cases, as Rainer Jaenicke discussed several years ago[17] .

Figure S1 Multiple alignment of the amino acid sequence from the 75 class A β-lactamases used in our ancestral sequence reconstruction. Residue numbering for enzymes follows Ambler et al[18]. Only the mature protein sequences are shown here. Analyses were actually carried out with the preprotein sequences which included the ~20 amino acids long signal peptide. However, signal peptides have been omitted here because they show little sequence similarity and their alignment includes large numbers of gaps. Insertions that occur in very small numbers of sequences have also been removed (see "Materials and Methods" for details).



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gi27468526    APY NKL NKK VHI NKD DIV AYS PIL EKY VGK DIT LKE LIE ASM KYS DNT ANN KII NEI GGI
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gi15609205        AGGDAEFREALLAEAATCVAGVLA---
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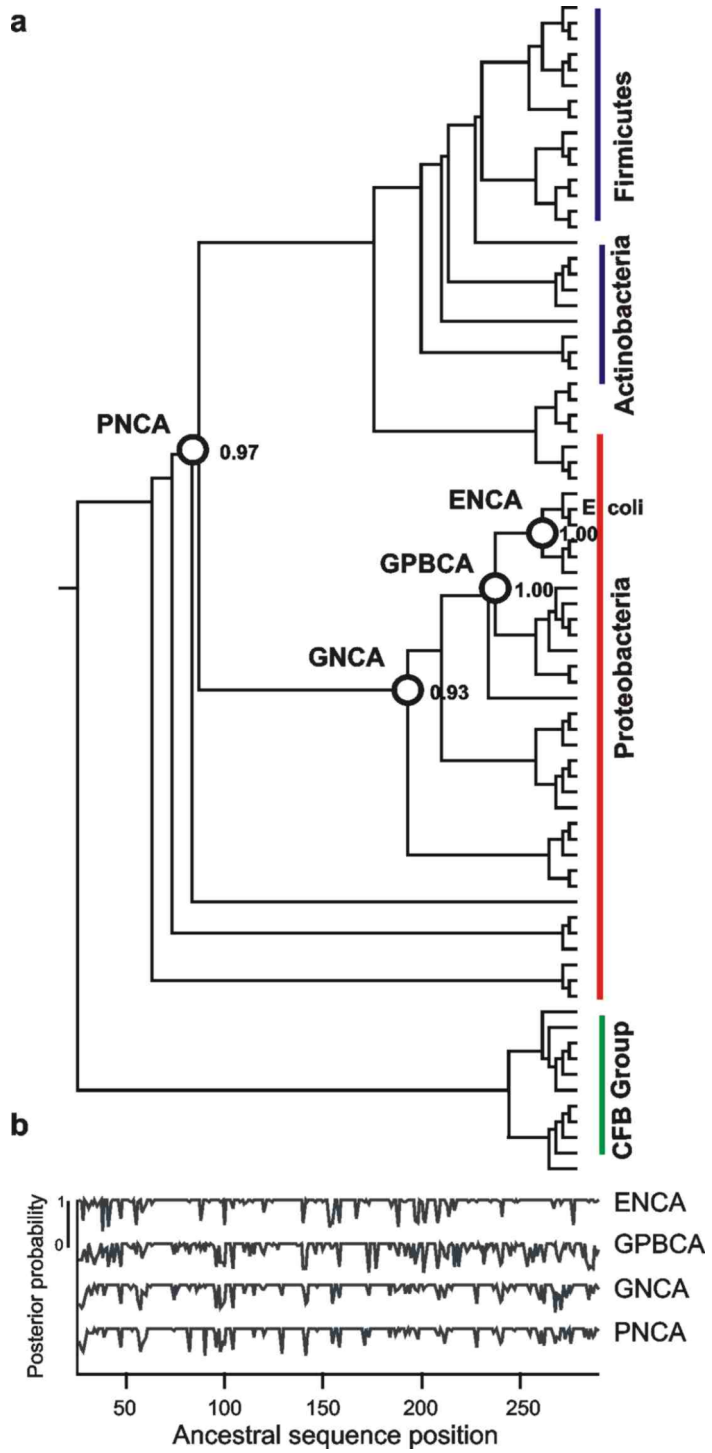

Figure S2 Phylogenetic analysis of β-lactamases and ancestral sequence reconstruction. (a) Cladogram of the tree used as a basis for the laboratory resurrection of Precambrian β-lactamases. The internal nodes targeted are labeled and their posterior probabilities shown. **(b)** Posterior probability profiles of the inferred amino acids across the 262 positions for the targeted internal nodes. The amino acid incorporated at each position of the ancient protein is that with the highest posterior probability.

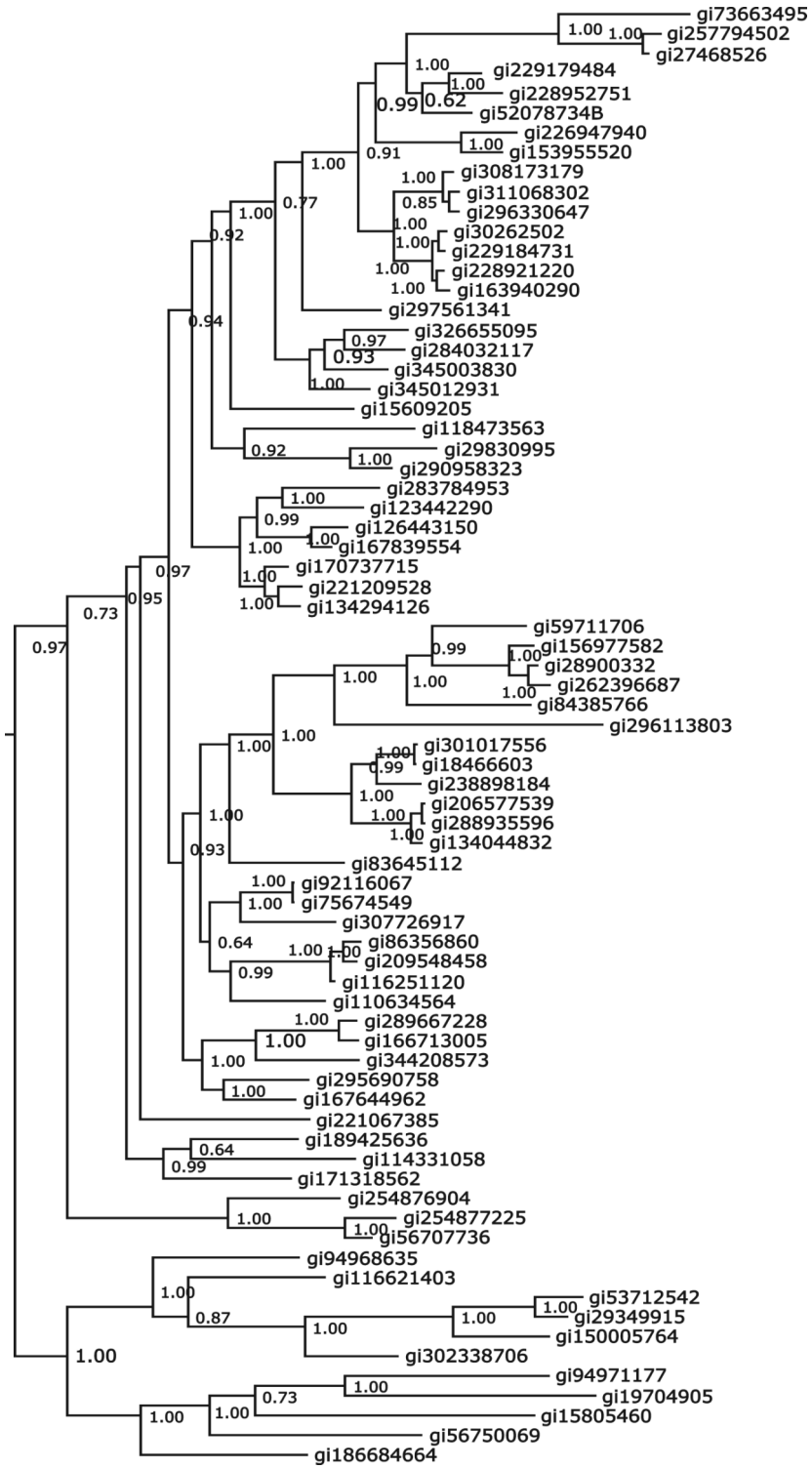

Figure S3 Cladogram of the tree used as a basis for the reconstruction of the sequences of Precambrian β-lactamases. This tree is identical with that in Figure S2, except that the posterior probabilities indicating nodal support are shown for all nodes and the GI numbers for the extant sequences are included (see Table S1). Branch lengths are poportional to distances as measured in amino acid substitutions.

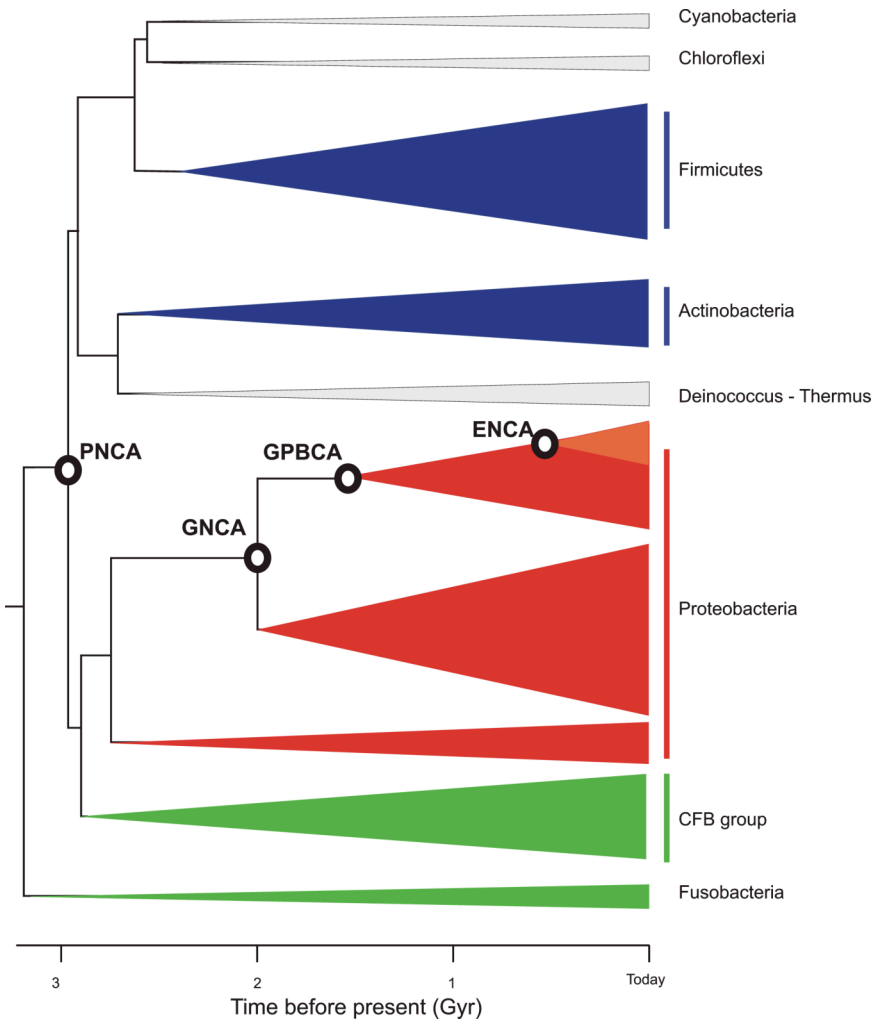

Figure S4 Schematic representation of the tree for Eubacteria given in the Time Tree of Life. The Precambrian nodes targeted are labeled and geological time scale has been added.

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TEM-1      A GQE QLG RRI HYS QND LVE YSP VTE KHL TDG MTV REL CSAA IT MSD NTAA NLLL TT IGG P
ENCA       A GLE QLD RRI HYR QQD LVE YSP VTE KHL ADG MTV AEL CAAA IT MSD NTAA NLLL A TIGG P
GPBCA      A GKE NLD RRI SYR KED LVD YSP VTE KHV GDG MTV AEL CEAA IT MSD NTAA NLLL E AIGG P
GNCA       Q GKE RLD RRI TYG KED LVD YSP VTE KHV GDG MTV AEL CEAA IT LSD NTAA NLLL E ALGG P
PNCA       Q GKE RLD RRI TYS KAD LVD YSP VTE KHV GGG MTV AEL CEAA IT YSD NTAA NLLL E ALGG P
           * *.*.*** * : ***:********: .**** *** **** ********** ::***

TEM-1      K ELT AFL HNM GDH VTR LDR WEP ELN EAI PNDE RD TTM PVA MATT LR KLL TGE LLT LAS RQ
ENCA       A GLT AFL RNI GDN VTR LDR WET ELN EAL PGDE RD TTT PAA MAA TLR KLL TGE ILS AAS RQ
GPBCA      A GLT AFL RSI GDE VTR LDR WEP ELN EAA PGD PRD TTT PAA MAA TLR TLLL GD VLS PAS RQ
GNCA       AA LT AFL RSI GDE VTR LDR WEP ELN EAA PGD PRD TTT PAA MAA TLR TLLL GD ALS PAS RQ
PNCA       AA LT AFL RSI GDE VTR LDR WEP ELN EAL PGD PRD TTT PRA MAA TLR KLLL G DAL SPA SRE
            *****:.:**.********.***** *.* **** * ***:***.** *: *: ***:

TEM-1      Q LID WME ADK VAG PLL RSA LPA GWF IAD KSG AGE RGS RG IIAA LG PDG KPS RIVVI Y TTG
ENCA       Q LIT WMV ADK VAG PLL RSVL PA GWF IAD KTG AG ERG SRG IIA VLG PDG KPS RIVVI Y LTE
GPBCA      Q LVA WLV ANK TGD KRL RAG LP ADW RIG DKT GAGG YG STN DIA VIW PPG RAP IIV TVY LTE
GNCA       Q LVD WLV ANK TG DKR LRA GLP ADW RVG DKT GTGG HG TTN DIA VIW PPG RAP IVV TVY LTE
PNCA       Q LVD WLL AN KTG DKR LRA GLP ADW RVG DKT GTGG HG TTN DIA VIW PPN RAP IVV AVYY TE
          **: *: *:*...  **: ***.* :.**:*:*  *: . **.: * .:.. :*.:* *

TEM-1      S QAT MDE RN RQI AEI GAS LIK HW
ENCA       T QAS MDE RN QQI AEI GAA LIE HW
GPBCA      T QAS MDE RN KVI AEV GRA VVE AF
GNCA       S QVD AD ARD AVI AEV GRLVV E AF
PNCA       S QAD AE ARD AVI AEV GRLV AE AF
          :*.  : *:  ***:*  :     : :
```

Figure S5 Sequence of TEM-1 β-lactamase and reconstructed sequences for the targeted Precambrian nodes (see Figures S2 and S4). The reconstructed sequences shown are the most probabilistic from each node (i.e., the sequences with the most probable amino acid at each position).

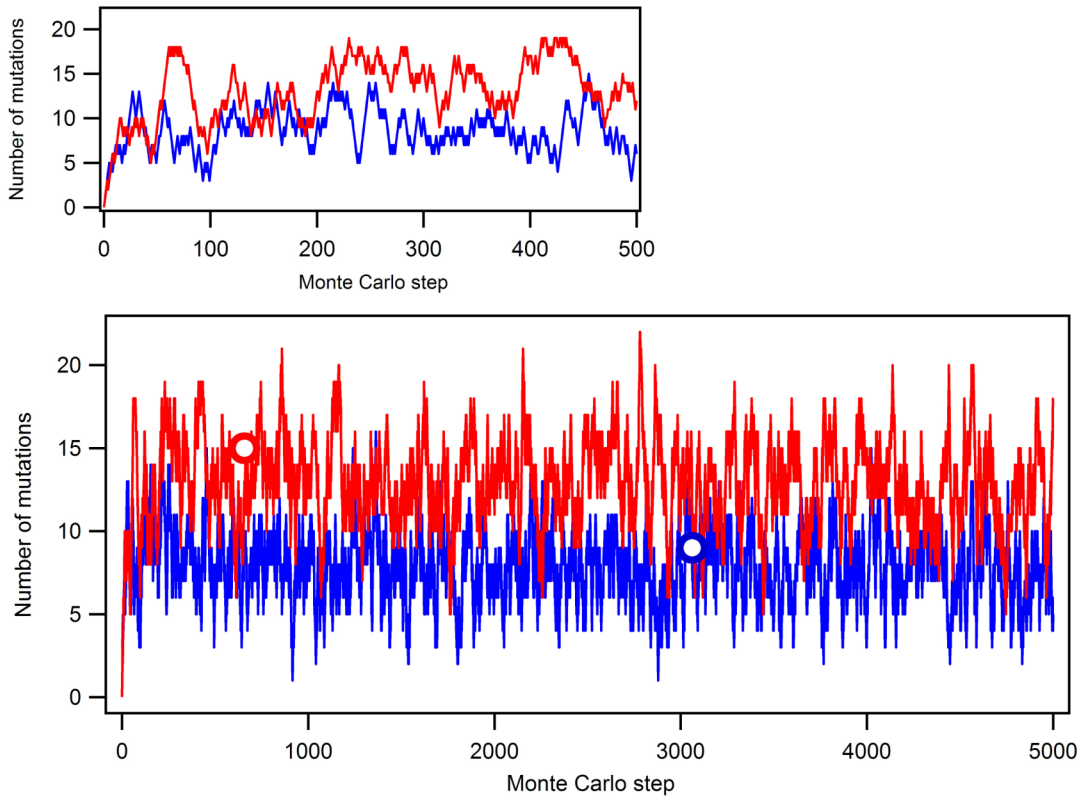

Figure S6 Sequence Monte Carlo sampling based on the posterior amino acid probability distribution for the GNCA node. At each step, a position and amino acid are randomly selected and a replacement to a particular amino acid is accepted or rejected according to its posterior probability at the selected position. This procedure may occasionally introduce very-low probability mutations which are likely to be disruptive (and which would be rejected during the course of natural evolution). To prevent this from taking place, a probability threshold is imposed in the Monte Carlo runs, in such a way that mutations with probabilities below the threshold are always rejected. Thresholds were actually defined in terms of the ratio between the probability and the highest probability at the position. The Monte Carlo runs shown were obtained with ratios of 0.5 (blue) and 0.2 (red) and were used to obtain alternative sequences at the GNCA node in order to test phenotypic robustness (see Table S4 and Figure S9). These sequences were derived from the steps labeled with circles and are shown in Figure S7.

```
GNCA-0     AAQLSEQLAELEKRSGGRLGVAVLDTATGRRIAYRGDERFPMCSTFKALLAAAVLARVDQ
GNCA-1     ASQLSEQLAELEKRSGGRLGVAVLDTATGRRIAYRGDERFPMCSTFKALLAAAVLARVDQ
GNCA-2     AAALSEQLAELEKRSGGRLGVAVLDTATGRRFGYRGDERFPMCSTFKALLAAAVLARVDQ
           *: ****************************:.****************************

GNCA-0     GKERLDRRITYGKEDLVDYSPVTEKHVGDGMTVAELCEAAITLSDNTAANLLLEALGGPA
GNCA-1     GKERLDRRITYSKADLVDYSPVTEKHVGDGMTVAELCEAAITMSDNTAANLLLESLGGPA
GNCA-2     GKENLDRRITYGKEDLVDYSPVTEKHVGDGMTVAELCEAAITYSDNTAANLLLEALGGPA
           ***.*******.* **************************** ***********:*****

GNCA-0     ALTAFLRSIGDEVTRLDRWEPELNEAAPGDPRDTTTPAAMAATLRTLLLGDALSPASRQQ
GNCA-1     ALTAFLRSIGDEVTRLDRWEPELNEAAPGDPRDTTTPAAMAATLRTLLLGDALSPASRQQ
GNCA-2     ALTAFLRSIGDNVTRLDRWEPELNTAAPGDPRDTTTPAAMAATLRTLLLGDVLSPASRQQ
           ***********:************ **************************.********

GNCA-0     LVDWLVANKTGDKRLRAGLPADWRVGDKTGTGGHGTTNDIAVIWPPGRAPIVVTVYLTES
GNCA-1     LVDWLIANKTGDKRLRAGLPADWRVGDKTGTGGHGTTNDIAVIWPPGRAPIVVTVYLTEA
GNCA-2     LVDWLIANKTGDKRLRAGLPADWRVGDKTGTGEHGTTNDIAVIWPPNRAPILVAVYLTES
           *****:*************************** *************.****:*:*****:

GNCA-0     QVDADARDAVIAEVGRLVVEAF
GNCA-1     QADADARDAVIAEVARLVVEAF
GNCA-2     QVDADARDAVIAEVARLVVAAF
           *.************.**** **
```

Figure S7 Reconstructed sequences of β-lactamases corresponding to the GNCA node. The most probabilistic sequence is shown (GNCA-0), together with the sequences of two alternative reconstructions (GNCA-1 and GNCA-2) derived from a Monte Carlo sampling based on the posterior amino acid distribution at the node (see Figure S6). The number of amino acid differences between GNCA-1 and GNCA-2 sequences compared with the GNCA-0 are 9 and 15 sites, respectively. Experimental measurements on the proteins corresponding to these three sequences were used to test the phenotypic robustness of the laboratory resurrection process (see Table S4 and Figure S9).

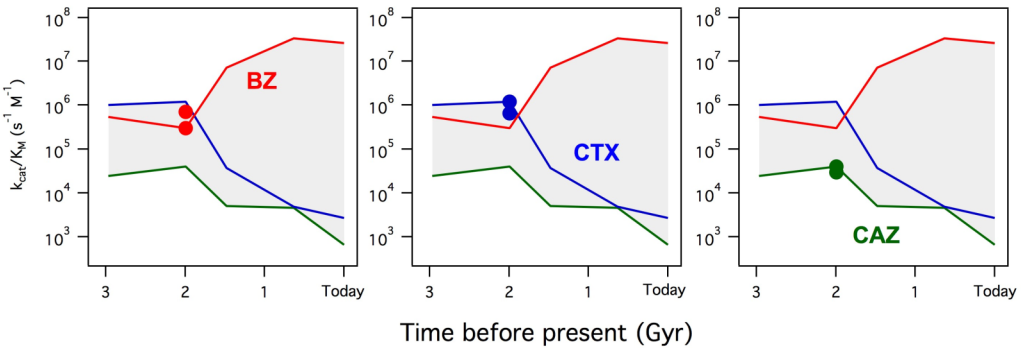

Figure S8 . Second-order rate constant for the in vitro hydrolysis of antibiotics catalyzed by three alternative reconstructions (GNCA-0, GNCA-1 and GNCA-2: see Figure S7) at the GNCA node. The data are plotted in a schematic representation of Figure 3A in the main text to emphasize that the uncertainty associated to the reconstruction does not signficantly affect the overall phenotypic picture.

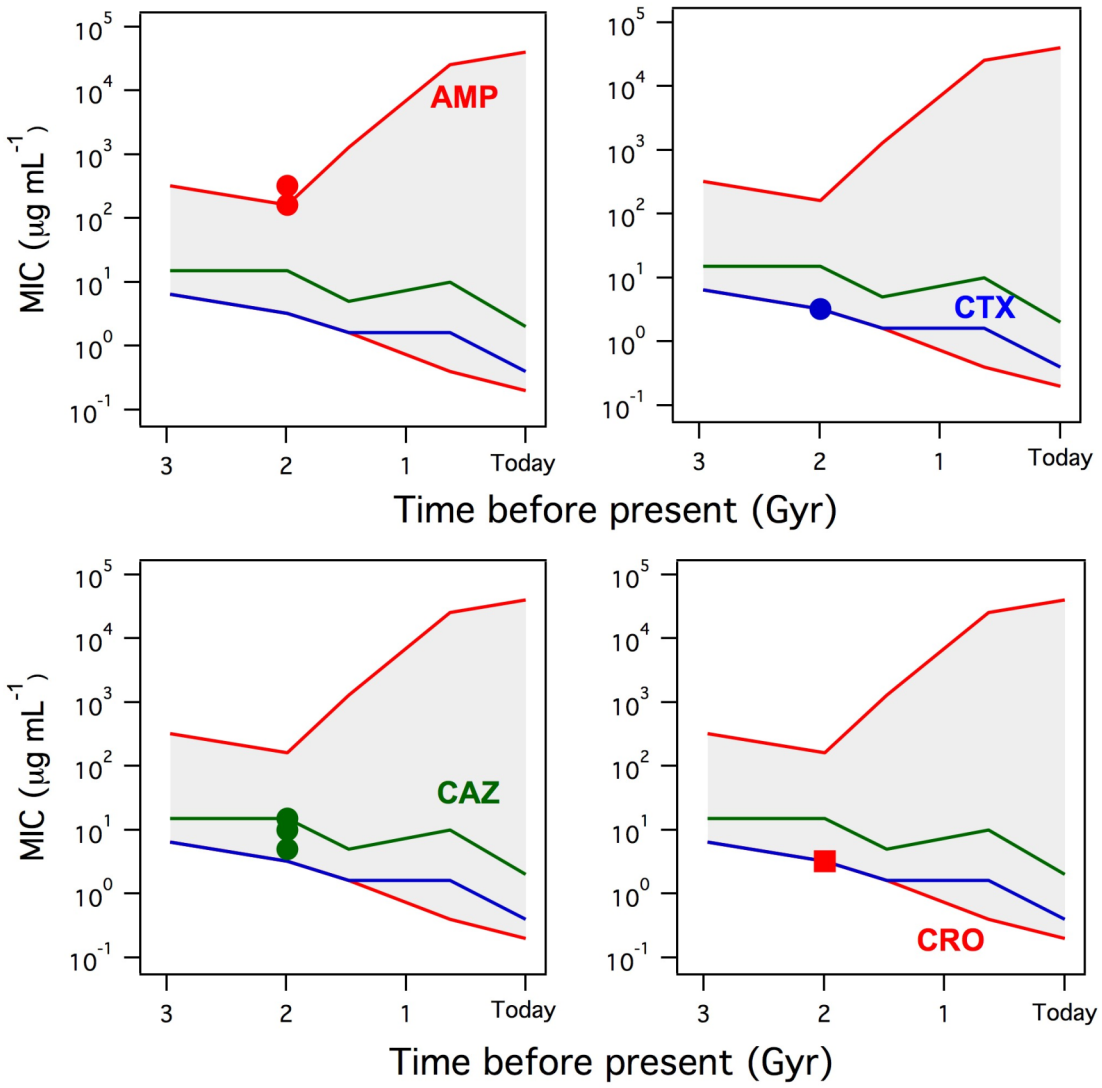

Figure S9 MIC values determined using an *E. coli* strain tranformed containing three alternative reconstructions (GNCA-0, GNCA-1 and GNCA-2: see Figure S7) at the GNCA node. The data are plotted in a schematic representation of Figure 3D in the main text to emphasize that the uncertainty associated to the reconstruction does not signficantly affect the overall phenotypic picture.

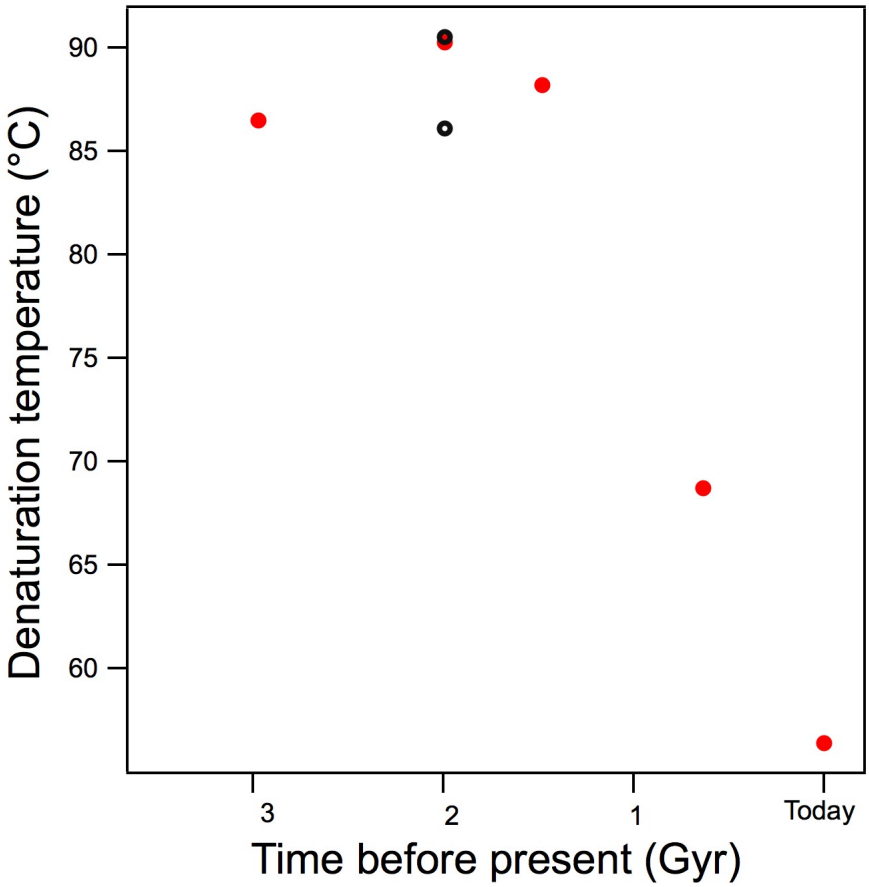

Figure S10 Denaturation temperature values for three alternative reconstructions (GNCA-0, GNCA-1 and GNCA-2: see Figure S7) at the GNCA node. The data are plotted in a schematic representation of Figure 2A in the main text to emphasize that the uncertainty associated to the reconstruction does not signficantly affect the overall phenotypic picture.

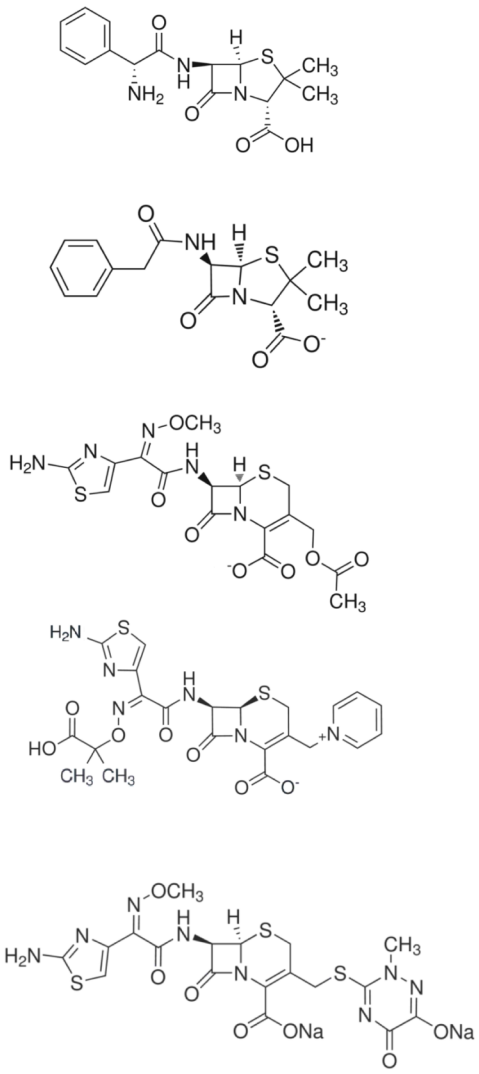

Figure S11 Chemical structures of the β-lactam substrates used in this work.

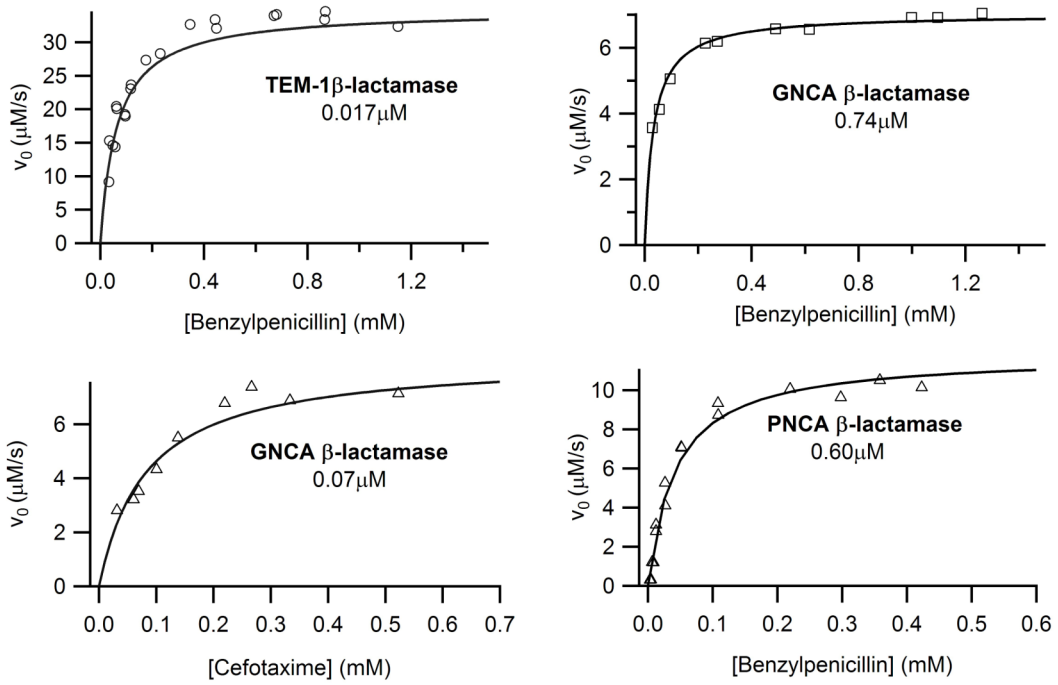

Figure S12 Catalysis of *in vitro* hydrolysis of antibiotics by extant and laboratory resurrections of Precambrian β-lactamases. Representative plots of initial rate versus substrate concentration. The rate values were derived from the time dependence of the UV absorbance associated with antibiotic hydrolysis. For experiments at cefotaxime concentrations > 250 μM, 0.2 cm pathlength cells were used to avoid absorbance values that were too high. The continuous lines are the best fits of the Michaelis-Menten equation. The values obtained for the Michaelis-Menten parameters are collected in Tables S4 and S5.

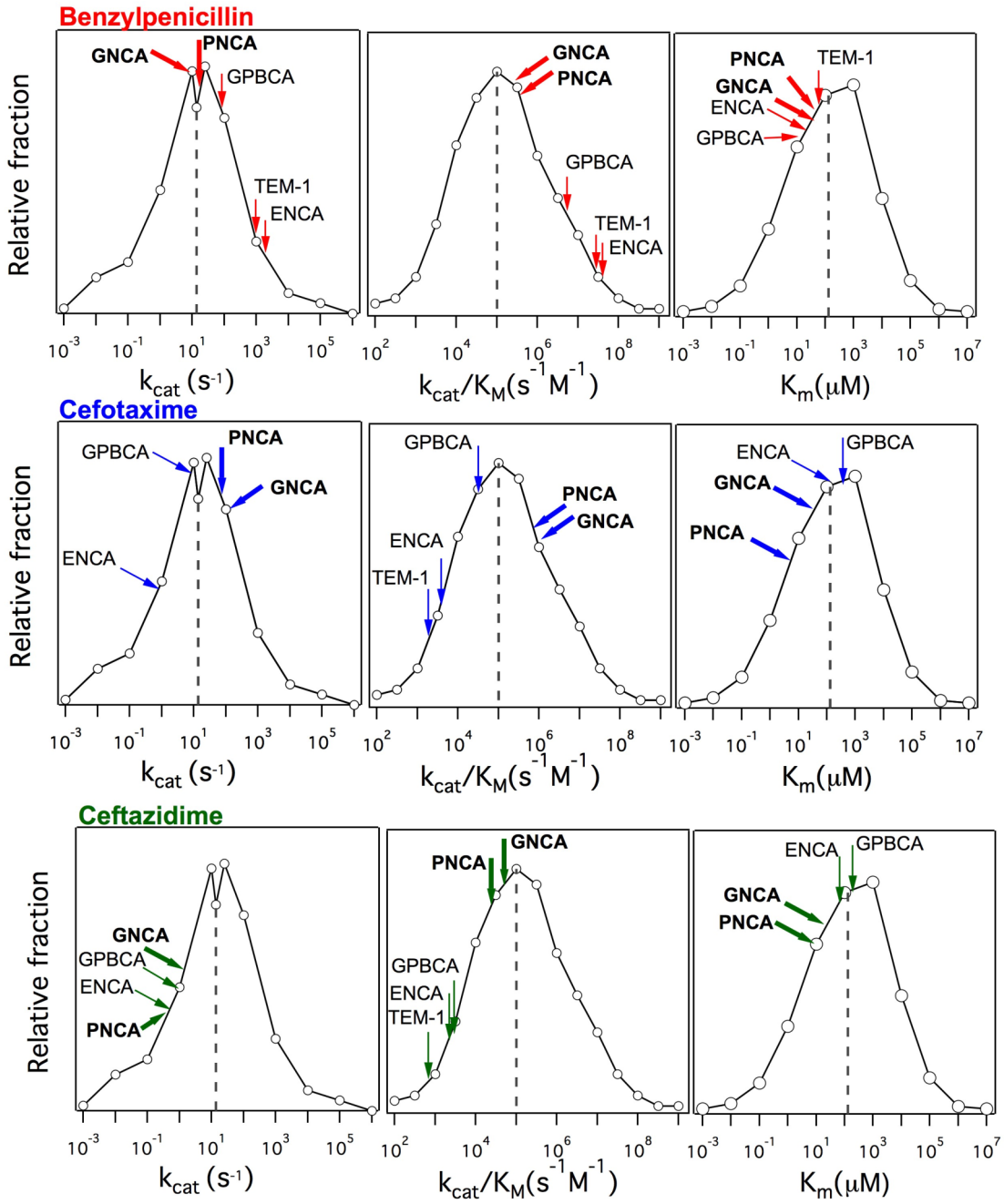

Figure S13 Distribution of kinetics parameters for moderm enzymes with added arrows that locate the values for the TEM-1 and the resurrected β-lactamases. Open circles represent values taken from Bar-Even et al. (ref. 15 in the main text). The antibiotics studied are: benzylpenicillin, and the third-generation cefotaxime and ceftazidime (Figure S11). Thicker arrows are used for the laboratory resurrections of the 2-3 billion-years-old promiscuos lactamases.

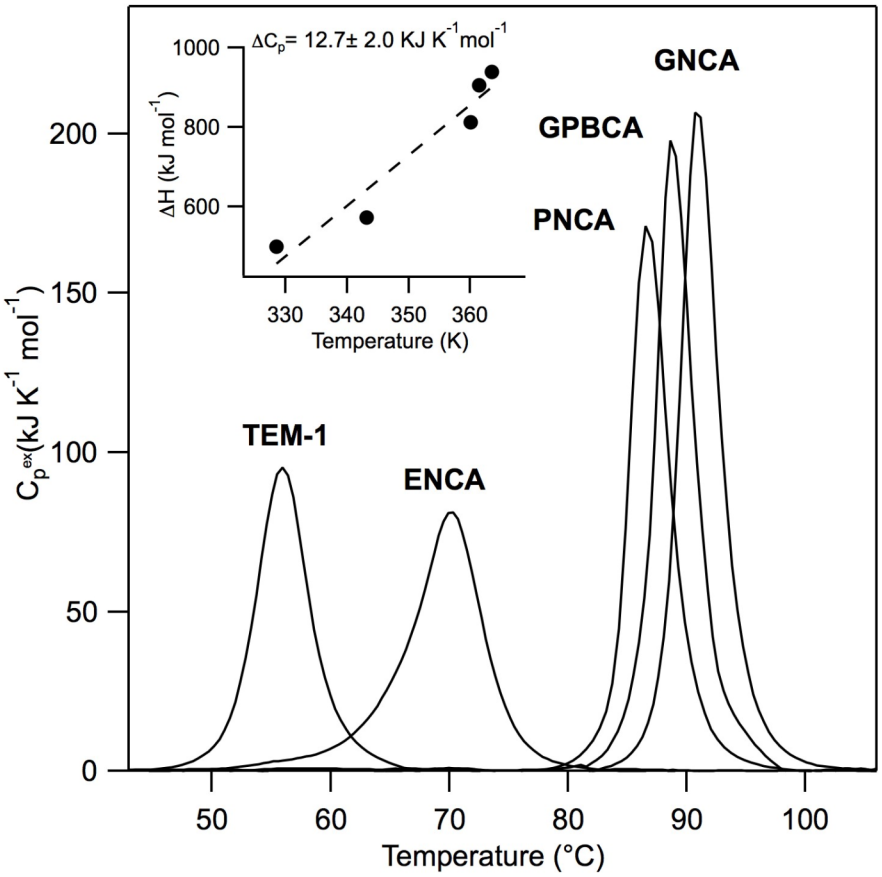

Figure S14 Thermal denaturation of extant and laboratory resurrected Precambrian β-lactamases as followed by differential scanning calorimetry (DSC). The DSC profiles shown were obtained at a scan-rate of 200 K/hour and a protein concentration of 1 mg/mL. Additional experiments within the range 0.1-1.5 mg/mL indicated the absence of a significant concentration dependence of the DSC profiles. On the other hand, experiments at different scanning rates within the range 60-200 K/hour did reveal changes in denaturation temperature (T_m). These scan-rate effects on T_m are significant, in particular for the more recent β-lactamases (see Figure S15), but they are still much smaller that the differences in T_m between the extant and laboratory resurrections of Precambrian β-lactamases. The difference in transition enthalpy (area under the heat capacity profile) visually apparent in the plots shown here is due to the increase in denaturation enthalpy with temperature associated with a positive value of the unfolding heat capacity change. Actually, a plot of denaturation enthalpy versus denaturation temperature (inset) gives a slope consistent with the unfolding heat capacity change expected for a protein of the size of β-lactamase.

Denaturation temperature values given in Fig 2A for the TEM-1 and Precambrian lactamases are actually derived from the transitions shown here. T_m values for the extant β-lactamases from *B. licheniformis* (unpublished work from our lab), *E. cloacae*[19] and *B. cereus*[20] are also included in Fig 2A. *B. licheniformis* is a thermophilic organims, which accounts for its somewhat enhanced Tm value (see Fig. 2A) which is, nevertheless, about

25 degrees below the Tm values for the GNCA β-lactamase. Published kinetic parameters [21] support that the β-lactamase from *B. licheniformis* is a pencillin specialist.

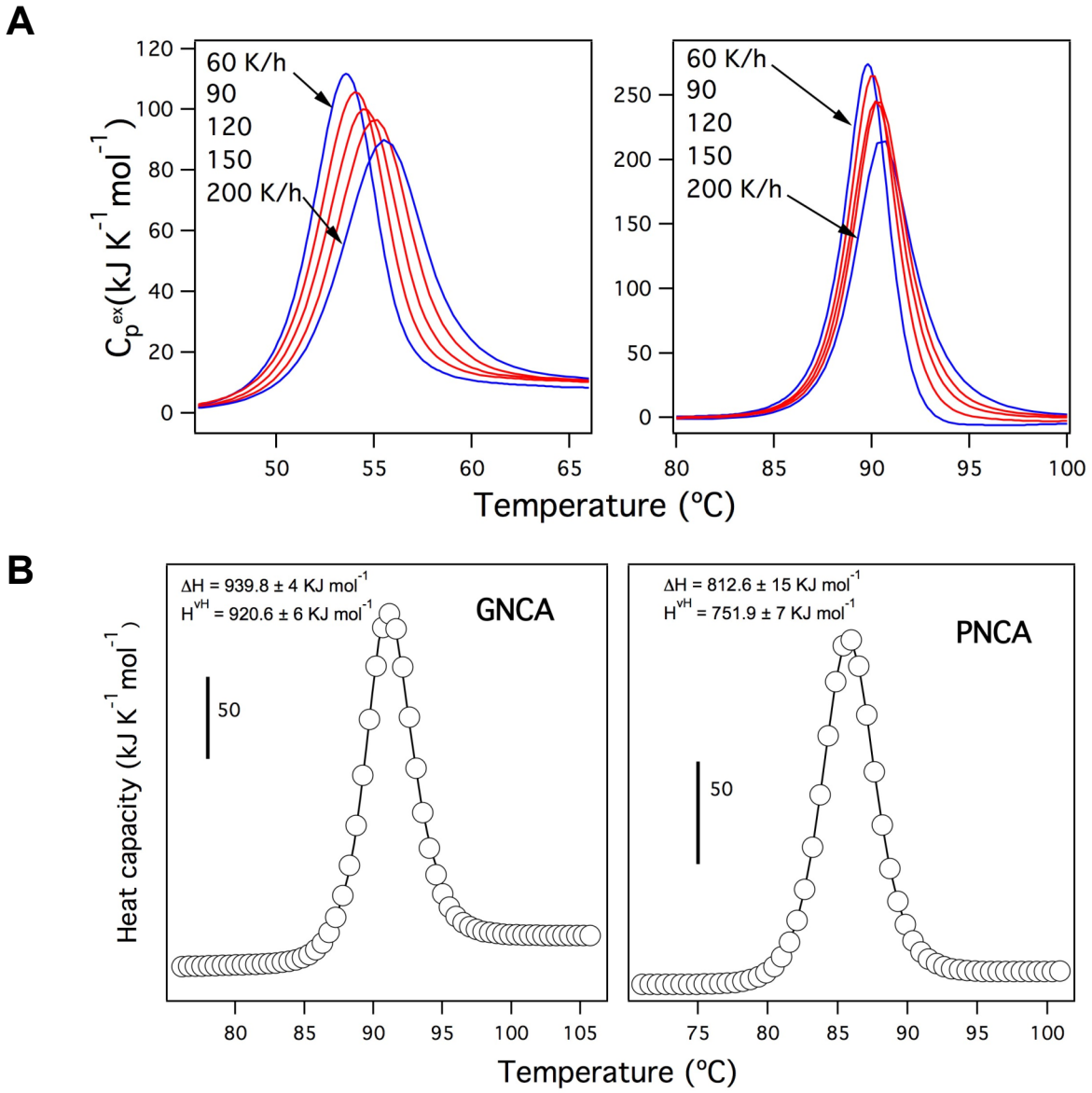

Figure S15 Thermal denaturation of extant and laboratory resurrected Precambrian β-lactamases as followed by differential scanning calorimetry (DSC). (A) Representative DSC profiles illustrating the effect of scanning rate (left panel: TEM-1, right panel: GNCA). (B) Fits of a pseudo two-state model to the DSC profiles corresponding to the GNCA and PNCA β-lactamases. Circles are the experimental data and the continuous line represents the best fits. The values of the calorimetric and van't Hoff enthalpy values derived from the fits are shown. Note that the agreement between the two enthalpy values supports the adequacy of the two-state model. The DSC profiles analyzed are those obtained at a scan-rate of 200 K/hour, since a high scan-rate minimizes kinetic distortions[12]

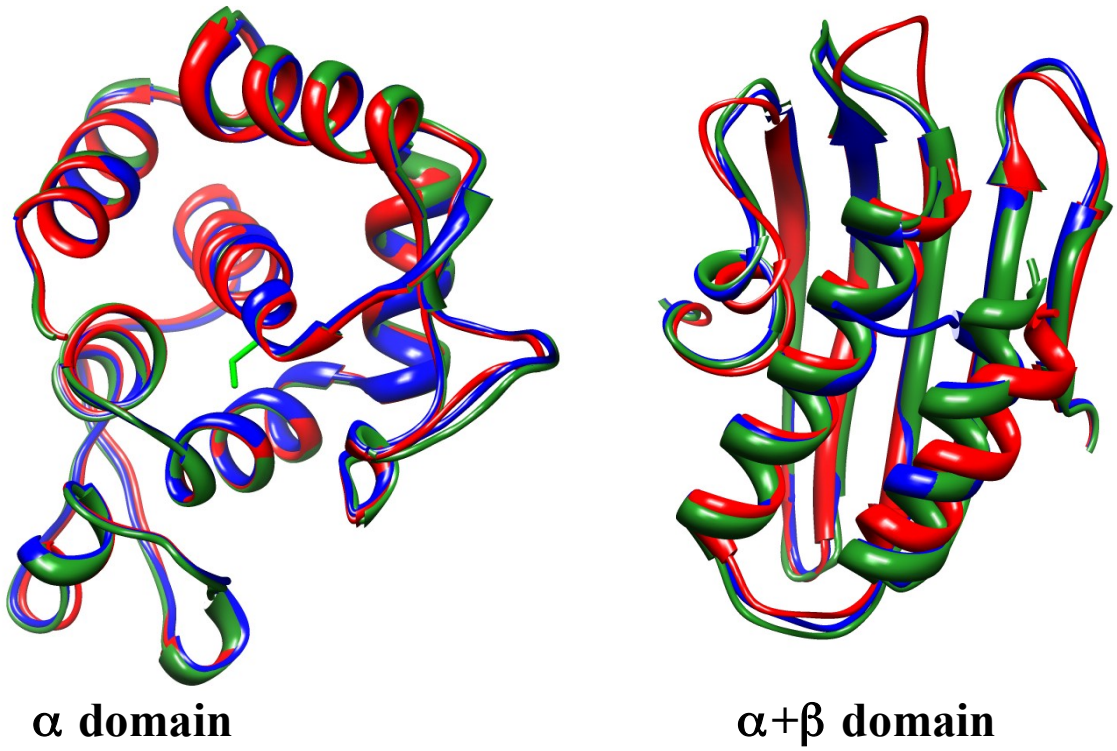

Figure S16 Structural comparison of the α and α+β domains of TEM-1 β-lactamase from *E. coli* (PDB.ID 1btl; green) and the laboratory resurrected β-lactamase corresponding to the last common ancestor of Gram-negative bacteria (GNCA) (PDB.ID 4b88; red) and the last cammon ancestor of enterobacteria (ENCA) (PDB.ID 3zdj; blue). Note that the differences are mostly confined to the α+β domain, while little disparity is observed in the active-site α domain.

Table S1 Sequences of extant β-lactamases class A used for ancestral sequence reconstruction. GI numbers were accessed from the GenBank. The names of the hosting organisms are also provided. Numbers are the amino acid length of the preprotein and the mature protein sequence in the alignment of Figure S1. A pubmed search (lactamase class A "organism name") yields specificity information for only 4 of the extant enzymes: GI301017556 (Frère JM. Mol Microbiol. (1995);(3):385-95), GI52078734 (Brown NG, Pennington JM, Huang W, Ayvaz T, Palzkill T. J Mol Biol. (2010);404(5):832-46), GI257794502 (Gebhard LG, Risso VA, Santos J, Ferreyra RG, Noguera ME, Ermácora MR. J Mol Biol. (2006) ,358(1):280-8) and GI56707736 (Antunes NT, Frase H, Toth M, Vakulenko SB. Antimicrob Agents Chemother. (2012) ;56(2):666-71). These 4 enzymes are reported to have high activity for penicillins and low activity for other antibiotics.

GI 110634564 Chelativorans (290/264)
GI 114331058 Nitrosomonas (319/263)
GI 116251120 Rhizobium (304/262)
GI 116621403 Solibacter (281/257)
GI 118473563 Mycobacterium (293/263)
GI 123442290 Yersinia (294/263)
GI 126443150 Burkholderia (314/263)
GI 134044832 Yersinia (286/265)
GI 134294126 Burkholderia (297/263)
GI 150005764 Bacteroides (319/264)
GI 153955520 Clostridium (310/264)
GI 15609205 Mycobacterium (303/261)
GI 156977582 Vibrio (283/265)
GI 15805460 Deinococcus 1 (265/253)
GI 163940290 Bacillus (309/261)
GI 166713005 Xanthomonas (302/266)
GI 167644962 Caulobacter (300/265)
GI 167839554 Burkholderia (294/263)
GI 170737715 Burkholderia (312/263)
GI 171318562 Burkholderia (305/264)
GI 18466603 Salmonella (286/263)
GI 186684664 Nostoc (339/263)
GI 189425636 Geobacter (300/266)
GI 19704905 Fusobacterium (264/259)
GI 206577539 Klebsiella 342 (286/265)
GI 94971177 Koribacter (293/259)
GI 209548458 Rhizobium (296/262)
GI 221067385 Comamonas (301/265)
GI 221209528 Burkholderia (302/263)
GI 226947940 Clostridium (308/264)
GI 228921220 Bacillus (312/261)
GI 228952751 Bacillus (320/264)
GI 229179484 Bacillus (301/264)
GI 229184731 Bacillus (312/261)
GI 238898184 Hamiltonella (286/265)

GI 254876904 Francisella (291/265)
GI 254877225 Francisella (287/265)
GI 257794502 Staphylococcus (281/260)
GI 262396687 Vibrio (283/265)
GI 27468526 Staphylococcus (284/260)
GI 283784953 Citrobacter (295/262)
GI 284032117 Kribbella (268/262)
GI 288935596 Klebsiella (286/265)
GI 28900332 Vibrio (283/265)
GI 289667228 Xanthomonas (297/264)
GI 290958323 Streptomyces (296/264)
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GI 295690758 Caulobacter (296/264)
GI 296113803 Moraxella (309/259)
GI 296330647 Bacillus (304/261)
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GI 308173179 Bacillus (304/261)
GI 311068302 Bacillus (304/261)
GI 326562148 Moraxella (307/257)
GI 326655095 Streptomyces (312/262)
GI 344208573 Burkholderia (304/264)
GI 345003830 Streptomyces (311/261)
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GI 52078734 Bacillus (307/264)
GI 53712542 Bacteroides (300/ 265)
GI 56707736 Francisella (287/265)
GI 56750069 Synechococcus (310/257)
GI 59711706 Vibrio (283/266)
GI 73663495 Staphylococcus (290/260)
GI 75674549 Nitrobacter (296/263)
GI 83645112 Hahella (299/264)
GI 84385766 Vibrio (319/265)
GI 86356860 Rhizobium (296/262)
GI 92116067 Nitrobacter (297/263)
GI 94968635 Koribacter (291/265)

Table S2 Sequence differences between the 75 class A β-lactamases used in sequence reconstruction. For each lactamase, the differences with all the extant lactamases included in our analyses are shown in the order specified below. Sequence differences are given as number of aminoacids (total length is 262 amino acids).

Seq->	gi301017556	gi257794502	gi27468526	gi73663495	gi308173179
	gi30262502	gi311068302	gi229179484	gi52078734	gi229184731
	gi296330647	gi228921220	gi228952751	gi163940290	gi238898184
	gi94968635	gi94971177	gi116621403	gi226947940	gi153955520
	gi189425636	gi56750069	gi186684664	gi15805460	gi118473563
	gi15609205	gi29830995	gi326655095	gi290958323	gi345003830
	gi345012931	gi297561341	gi254876904	gi254877225	gi56707736
	gi59711706	gi156977582	gi28900332	gi262396687	gi84385766
	gi296113803	gi283784953	gi206577539	gi288935596	gi92116067
	gi75674549	gi284032117	gi18466603	gi134044832	gi123442290
	gi326562148	gi83645112	gi289667228	gi166713005	gi221067385
	gi171318562	gi170737715	gi221209528	gi126443150	gi307726917
	gi344208573	gi167839554	gi134294126	gi114331058	gi295690758
	gi167644962	gi86356860	gi209548458	gi116251120	gi110634564
	gi53712542	gi29349915	gi150005764	gi302338706	gi19704905

gi301017556	ID	180	182	192	157	164	164	174	171	165	164
165	179	165	69	192	212	198	177	168	163	213	204
203	166	174	169	166	167	162	167	177	188	197	202
155	164	156	155	163	180	164	89	91	140	140	165
2	87	168	179	142	161	165	164	161	157	156	156
140	159	152	161	162	162	148	151	149	151	153	212
217	214	209	217								
gi257794502	180	ID	25	132	156	157	155	150	154	157	155
160	155	157	189	203	214	206	160	161	188	217	207
214	182	177	190	172	188	164	163	162	187	189	181
183	191	181	186	187	190	174	193	194	184	186	162
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191	197	172	181	191	186	185	189	187	185	192	211
210	212	200	213								
gi27468526	182	25	ID	128	158	154	157	151	155	153	155
161	158	157	188	205	214	206	160	158	192	217	207
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191	197	174	182	190	184	185	188	187	184	191	211
214	214	202	211								
gi73663495	192	132	128	ID	167	168	164	157	162	167	163
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gi308173179	157	156	158	167	ID	76	23	104	112	78	22
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204	162	138	157	115	155	125	118	114	179	188	185
178	180	174	175	176	186	145	165	167	150	150	123
156	167	152	185	163	171	174	172	159	146	143	149
157	174	146	148	163	168	161	157	155	149	157	206
208	207	203	215								

gi30262502	164	157	154	168	76	ID	78	111	107	8	76
23	102	31	161	195	203	194	125	119	164	217	195
205	169	139	163	115	150	129	115	113	189	186	186
171	174	172	172	168	187	148	165	166	150	151	125
163	167	160	186	159	175	176	173	156	144	141	148
162	178	144	147	166	167	160	160	153	153	160	207
210	211	204	214								
gi311068302	164	155	157	164	23	78	ID	105	113	80	19
81	106	77	171	194	211	197	129	120	170	216	203
206	167	145	163	117	160	128	120	120	179	186	183
184	183	176	179	178	184	152	169	170	157	157	124
163	170	156	183	167	174	176	175	162	153	148	155
160	178	154	153	169	170	165	162	158	155	164	208
212	210	205	216								
gi229179484	174	150	151	157	104	111	105	ID	61	113	106
110	62	109	172	194	208	198	118	115	167	208	197
203	153	145	169	126	160	133	125	118	177	178	179
179	177	177	178	178	188	149	179	180	154	155	135
173	180	161	187	159	172	173	170	162	154	145	152
168	174	154	157	172	169	161	160	156	155	157	204
206	211	200	209								
gi52078734	171	154	155	162	112	107	113	61	ID	109	114
105	81	101	171	199	203	202	134	131	169	204	198
204	158	140	168	124	158	124	124	110	184	182	187
183	184	182	182	186	189	153	177	177	156	153	140
170	179	158	188	158	176	175	175	167	153	141	155
168	174	154	154	174	170	160	163	162	159	161	204
208	214	204	204								
gi229184731	165	157	153	167	78	8	80	113	109	ID	78
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206	170	138	162	116	151	130	116	113	190	185	183
169	173	172	172	169	188	149	166	167	151	152	126
164	168	157	187	159	174	177	173	157	146	141	148
162	177	145	148	166	168	161	160	154	153	161	206
209	211	206	213								
gi296330647	164	155	155	163	22	76	19	106	114	78	ID
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ID	145	136	137	132	136	128	115	119	114	129	203
208	209	197	217								
gi344208573	159	197	197	192	174	178	178	174	174	177	176
177	178	177	163	205	211	186	182	181	145	211	201
206	169	158	168	162	174	159	166	164	194	188	191
176	176	177	178	185	181	156	165	166	134	139	164
158	168	143	180	144	124	126	153	157	148	140	148
145	ID	144	146	149	140	134	156	153	149	146	210
215	217	214	226								

gi167839554	152	172	174	182	146	144	154	154	154	145	149
151	161	150	151	200	198	182	163	163	135	214	194
199	147	139	139	142	133	142	143	144	188	192	189
178	172	172	172	174	191	117	151	151	124	129	145
151	149	115	190	137	142	146	150	141	86	86	29
136	144	ID	88	153	142	142	147	147	139	137	196
200	203	205	218								
gi134294126	161	181	182	180	148	147	153	157	154	148	152
152	161	152	159	187	205	181	163	163	150	213	191
204	149	133	146	142	149	142	145	143	185	188	195
181	172	169	170	178	194	125	153	153	133	132	146
160	152	119	193	144	147	149	149	146	49	43	94
137	146	88	ID	151	145	143	147	142	141	139	206
209	213	206	227								
gi114331058	162	191	190	189	163	166	169	172	174	166	167
168	173	166	161	186	209	191	165	168	134	205	187
204	153	159	160	167	164	162	164	157	186	187	188
181	176	174	173	176	187	156	158	158	145	148	157
160	157	159	186	157	146	151	155	147	144	145	152
132	149	153	151	ID	149	146	150	151	149	151	204
208	203	199	213								
gi295690758	162	186	184	188	168	167	170	169	170	168	168
169	170	168	165	190	208	186	180	180	148	199	186
197	154	150	163	157	152	155	162	160	185	190	187
167	172	169	172	180	183	160	150	150	137	139	154
161	156	154	182	140	135	132	147	149	142	139	144
136	140	142	145	149	ID	109	135	132	133	142	207
211	209	209	219								
gi167644962	148	185	185	187	161	160	165	161	160	161	164
163	157	161	151	185	218	185	169	169	139	205	191
209	161	152	154	145	138	149	156	157	186	179	187
167	167	166	166	168	184	141	149	151	121	121	152
148	156	153	183	136	135	134	140	148	134	136	144
128	134	142	143	146	109	ID	124	123	122	123	199
209	214	200	218								
gi86356860	151	189	188	195	157	160	162	160	163	160	160
157	167	158	153	187	213	189	175	164	144	206	193
205	167	153	148	145	152	148	158	156	185	193	194
170	164	163	166	173	182	150	151	151	126	122	159
150	155	157	181	125	153	156	144	143	141	140	149
115	156	147	147	150	135	124	ID	28	27	126	200
206	204	198	218								
gi209548458	149	187	187	192	155	153	158	156	162	154	156
152	165	153	149	185	209	187	172	160	144	204	189
205	164	152	148	147	150	148	157	155	184	190	193
167	164	164	165	170	180	149	146	147	126	123	157
148	151	153	179	132	156	159	143	135	137	136	148
119	153	147	142	151	132	123	28	ID	23	122	199
202	202	199	218								
gi116251120	151	185	184	191	149	153	155	155	159	153	153
152	163	151	151	188	209	186	172	161	141	204	188
203	158	150	147	146	152	149	154	152	184	193	192
162	164	163	165	168	183	146	150	151	123	118	156
150	155	150	182	127	151	156	137	137	134	135	141
114	149	139	141	149	133	122	27	23	ID	118	199
204	204	203	219								

gi110634564	153	192	191	187	157	160	164	157	161	161	163
161	162	160	149	205	214	191	165	170	141	209	195
206	157	145	151	141	146	147	149	152	185	193	201
168	165	165	166	168	177	145	151	153	126	118	152
152	154	148	176	146	155	157	143	149	138	129	140
129	146	137	139	151	142	123	126	122	118	ID	208
209	210	203	223								
gi53712542	212	211	211	204	206	207	208	204	204	206	206
204	209	206	211	184	207	190	209	209	206	214	199
224	204	204	210	205	209	207	208	207	198	200	201
218	216	213	217	206	203	206	210	210	205	201	207
212	211	200	202	203	205	207	216	210	197	199	198
203	210	196	206	204	207	199	200	199	199	208	ID
61	130	166	210								
gi29349915	217	210	214	207	208	210	212	206	208	209	210
205	211	209	216	192	206	197	213	209	213	214	203
225	208	204	213	205	206	210	210	211	200	202	201
220	217	213	217	213	207	211	216	216	205	207	212
217	218	202	206	207	210	213	220	215	206	205	203
208	215	200	209	208	211	209	206	202	204	209	61
ID	119	163	210								
gi150005764	214	212	214	205	207	211	210	211	214	211	209
210	214	210	212	184	217	191	210	213	206	214	205
224	204	205	211	207	205	212	206	212	203	207	205
218	216	215	219	216	208	210	212	212	209	205	206
214	212	208	207	209	202	206	214	208	204	206	207
209	217	203	213	203	209	214	204	202	204	210	130
119	ID	155	207								
gi302338706	209	200	202	208	203	204	205	200	204	206	206
204	201	208	206	171	208	160	203	203	204	217	191
217	200	205	203	203	202	203	208	207	205	191	195
212	210	213	209	203	198	199	209	209	196	200	203
209	208	210	197	203	208	210	213	205	210	205	205
197	214	205	206	199	209	200	198	199	203	203	166
163	155	ID	210								
gi19704905	217	213	211	218	215	214	216	209	204	213	216
211	209	216	220	214	186	216	215	224	212	195	206
200	215	221	218	224	218	222	225	220	207	205	210
220	223	218	218	217	210	217	220	219	219	219	227
216	220	221	210	211	220	222	222	222	223	226	220
217	226	218	227	213	219	218	218	218	219	223	210
210	207	210	ID								

Table S3 Sequence differences between the β-lactamases studied in this work. This table includes the differences among extant TEM-1 β-lactamase, the laboratory resurrections of Precambrian β-lactamases, and also, for each lactamase, the difference with the best hit sequence (sequences with highest similarity with the query) obtained from BLAST searches in the non-redundant protein database. Sequence differences are given as number of aminoacids (total length is 262 amino acids). Diffrences of the sequences of the β-lactamases studied with the consensus sequence from the 75 class A β-lactamases used in our phylogenetic analysis are also provided; these differences with the consensus, however, are given as the percentage of ancestral vs. TEM-1 differences that differ from the consensus prediction.

Sequence Difference	**TEM-1**	**ENCA**	**GPBCA**	**GNCA**	**PNCA**
TEM-1	-	55	111	122	124
ENCA	55	-	79	98	104
GPBCA	111	79	-	30	43
GNCA	122	98	30	-	17
PNCA	124	104	43	17	-
Best hit	-	42	79	79	84
Consensus sequence	-	64%	41%	42%	39%

Table S4 Testing the phenotypic robustness of the laboratory resurrection of Precambrian β-lactamases. Denaturation temperatures (from DSC experiments at 200 K/h), MIC values and Michaelis-Menten parameters for the GNCA β-lactamases corresponding the most probabilistic sequence and two alternative Monte Carlo sampling reconstructions (Figures S6-S10). For comparison, data for the extant TEM-1 lactamase are also included.

	TEM-1	GNCA-0	GNCA-1	GNCA-2
T_m (°C)	55.37	90.39	90.51	86.10
Log_{10}(MIC μg ml^{-1})				
AMP	3.602	1.20	1.50	1.20
CTX	-1.39	-0.49	-0.49	-0.49
CAZ	-0.69	0.17	0	-0.30
CRO	-1.69	-0.49	-0.49	-0.49
Kinetic Parameters				
BZ				
k_{cat} (s^{-1})	1760±100	9.5±1.1	12.5±2	36±2
K_m (μM)	66±8	32±12	17±3	158±56
k_{cat}/ K_M (s^{-1} μM^{-1})	26±4.7	0.3±0.1	0.7±0.2	0.2±0.1
CTX				
k_{cat} (s^{-1})	ND	116±7	140±25	126±35
K_M (μM)	ND	97±20	114±15	197±26
k_{cat}/ K_m (s^{-1} μM^{-1})	$2.6 \times 10^{-3} \pm 1 \times 10^{-3}$	1.2±0.3	1.2±0.4	$6.4 \times 10^{-1} \pm 0.3$
CAZ				
k_{cat} (s^{-1})	ND	1.5±0.1	1.7±0.2	0.9±0.2
K_m (μM)	ND	37±8	56±7	75±13
k_{cat}/ K_M (s^{-1} μM^{-1})	$6.5 \times 10^{-4} \pm 3 \times 10^{-4}$	$40 \times 10^{-3} \pm 11 \times 10^{-3}$	$29 \times 10^{-3} \pm 7 \times 10^{-3}$	$11 \times 10^{-3} \pm 5 \times 10^{-3}$

Table S5 Michaelis-Menten parameters for the hydrolysis of several antibiotics catalyzed by the β-lactamases studied in this work. In all cases, three independent determinations were performed and the values given are the corresponding averages and standard deviations.

Substrates	Variants	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/ K_m (s^{-1} μM^{-1})
BZ	TEM-1	1760±100	66±8	26±4.7
	ENCA	878±38	26±4	34±6.6
	GPBCA	85±20	12±2	7±2
	GNCA	9.5±1.1	32±12	0.3±0.1
	PNCA	18.9±0.5	35±1	0.5±0.03
CTX	TEM-1	ND	ND	$2.6 \times 10^{-3} \pm 1 \times 10^{-3}$
	ENCA	0.77 ±0.1	160±39	$4.8 \times 10^{-3} \pm 1 \times 10^{-3}$
	GPBCA	8.9±1	240±34	$37 \times 10^{-3} \pm 9 \times 10^{-3}$
	GNCA	116±7	97±20	1.2±0.3
	PNCA	73±16	72±15	1.0±0.4
CAZ	TEM-1	ND	ND	$6.5 \times 10^{-4} \pm 3 \times 10^{-4}$
	ENCA	0.4±0.02	80±20	$4.6 \times 10^{-3} \pm 1 \times 10^{-3}$
	GPBCA	0.9±0.03	180±50	$5 \times 10^{-3} \pm 1 \times 10^{-3}$
	GNCA	1.5±0.1	37±8	$40 \times 10^{-3} \pm 11 \times 10^{-3}$
	PNCA	0.3±0.05	10±1	$30 \times 10^{-3} \pm 8 \times 10^{-3}$

Table S6 Minimum inhibitory concentration (MIC) values determined using an E. coli strain tranformed with plasmid containing the ancestral β-lactamases. The MICs of each transformant, as well as that of the control strain *E. coli* ATCC 25922, were assayed in triplicate with serially diluted (2-fold) antibiotic stocks. In all cases, identical values were obtained and, therefore, the variability between different experiments is less than twofold

	TEM-1	ENCA	GPBCA	GNCA	PNCA
$Log_{10}(MIC/\mu g\ ml^{-1})$					
AMP	3.60	3.40	2.11	1.20	1.51
CTX	-1.39	-0.79	-0.79	-0.49	-0.19
CAZ	-0.69	0	-0.30	0.17	0.17
CRO	-1.69	-1.40	-0.80	-0.49	-0.19

Table S7 X-ray data collection and refinement statistics.

	GNCA	ENCA
Wavelength (Å)	1.54	1.54
Resolution range (Å)	18.66 - 2.05 (2.12 - 2.05)	48.13 - 2.4 (2.49 - 2.4)
Space group	I 41	P 64
Unit cell	94.833 94.833 95.34 90 90 90	111.144 111.144 93.722 90 90 120
Total reflections	196012 (27973)	546677 (2678)
Unique reflections	26446 (2628)	25827 (1246)
Multiplicity	7.4 (7.2)	21.17 (20.93)
Completeness (%)	100.00 (100.00)	99.68 (100.00)
Mean I/sigma(I)	13.8 (3.5)	15.38 (1.73)
Wilson B-factor	19.73	34.2
R-factor	0.1266 (0.1699)	0.1599 (0.2649)
R-free	0.1656 (0.2066)	0.1786 (0.2799)
Number of atoms	4336	8644
macromolecules	2054	4185
ligands	9	4
water	164	171
Protein residues	260	526
RMS(bonds)	0.010	0.013
RMS(angles)	1.36	1.30
Ramachandran favored (%)	98	98
Ramachandran outliers (%)	0	0
Clashscore	5.57	7.43
Average B-factor	24.80	29.80
macromolecules	23.60	29.30
solvent	39.90	36.90

Statistics for the highest-resolution shell are shown in parentheses.

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