Supporting Information

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SI Materials and Methods.

Strains and Plasmids. Chromosomal gene deletions were performed using the λ-red recombinase system (1). The asparaginase genes *ansA*, *ansB*, *iaaA*, the aspartate aminotransferase gene *aspC*, and the tyrosine aminotransferase gene *tyrB* were deleted from the chromosome of *Escherichia coli* MC1061 [F^- Δ*(ara-leu) 7697* $[araD139]_{B/r}$ Δ*(codB-lacI)3 galK16 galE15* λ^- *e*14^- *mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2* (r^-m^+)] resulting in *E. coli* JC1. Primers used for the deletion of each gene are found in Table S2. Briefly, each primer pair was used to amplify a PCR fragment containing the kanamycin resistance cassette of pKD13. Subsequently, the linear PCR product was used to replace the entire ORF of the targeted gene on the MC1061 chromosome. Colonies containing the correct gene deletions were transformed with the FLP recombinase plasmid pCP20 to remove the kanamycin resistance marker, and the pCP20 was then cured from the resulting strain as described previously (1). The genes *ansA*, *ansB*, and *iaaA* were also deleted from the *E. coli* strain BL21 (DE3) (F^- *ompT gal dcm lon* $hsdS_B(rB^-mB^-)$ λ*(DE3* [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) chromosome, resulting in *E. coli* JC2, used to express the EcAII variants. Gene deletions were transferred to recipient strains via P1 transduction.

All plasmids and primers used in this study are described in Tables S1 and S2, respectively. PCR reactions were carried out using *Vent* DNA polymerase (New England Biolabs) and oligonucleotides were synthesized by Integrated DNA Technologies. The *ansB* gene (mature sequence only) was amplified from the genomic DNA of *E. coli* K12 using the primers ansBFor/ansBRrev, digested with *NdeI-BamHI*, and cloned into pET-28a to generate plasmid pHisEcAII. Subsequently, plasmid pPelBHisEcAII was generated through subcloning the *NcoI-BamHI* digested fragment from pHisEcAII into pET-26b. In addition, a plasmid coding EcAII-T12A was generated by PCR using the pPelBHisEcAII plasmid as template and the primer pair T12AFor/T7 term, resulting in plasmid pPelBHisT12A.

To construct vector pCTK, first, the *tet* promoter region from vector pASK75 was amplified using the primers tetFor/tetRev, digested with *Xba*I-*Nco*I, and ligated into pCDF-1b. Next, the kanamycin resistance cassette from vector pET-28a was amplified using the primers KanFor/KanRev, digested with *BglI-BmtI*, and then cloned into pCDF-1b as well, ultimately generating the final pCTK vector.

Plasmids pCTK-EcAII and pCTK-T12A were generated by subcloning the *NcoI-NotI* digested fragments of pPelBHisEcAII and pPelBHisT12A, respectively, into vector pCTK. Plasmids pCTK-G57A, pCTK-G57V, and pCTK-G57L were constructed using a two-step protocol based on the QuickChange methodology. In the first step, megaprimers for each G57 mutant were amplified by PCR from the plasmid pCTK-EcAII using the primer pair 5′-G57X(X = A,V,L)/3′-G57 mp. In the second step, the megaprimer was used in place of outside primers in a PCR reaction again using plasmid pCTK-EcAII as the template with the following cycling parameters: 95 °C-2 min, 16 cycles of 95 °C-30 s, 55 °C-1 min, 72 °C-10 min, and a final polishing step at 72 °C-15 min. Each product was then digested with *DpnI* for 1 h at 37 °C to eliminate the initial template plasmid.

In Silico Identification and Characterization of EcAII T-Cell Epitopes. The primary sequence corresponding to the mature region of EcAII was screened for putative T-cell epitopes using the Immune Epitope Database (IEDB) consensus prediction method. The 326 amino acid sequence was parsed into overlapping 15-mer peptide fragments and within each fragment, a 9-mer core region was identified and scored for predicted binding by a consensus percentile rank (CPR) in which a lower score (in arbitrary units) was indicative of a higher predicted binding affinity. Because the consensus method scoring is based on the outputs of individual major histocompatability complex (MHC)-II binding prediction methods, multiple 9mer cores were identified in some 15mer fragments. In these instances, the 9mer core selected was the one predicted by TEPITOPE (*Sturniolo*) (2), which served as the basis for ProPred (3)—the most accurate algorithm for epitope core identification among those evaluated by the developers of the IEDB consensus method (4). Binding was further evaluated for seven additional HLA-DR alleles which when taken with DRB1*0401 cover nearly 95% of human populations worldwide (5). Three 9mer core regions that were scored with a CPR falling within the lowest 10% of the parsed peptide fragments as determined for binding to DRB1*0401 (CPR < 2) and that further received equivalently low scores for at least one other DRB1 allele were selected for neutral drift combinatorial mutagenesis.

Library Construction. Oligonucleotides encoding degenerate NNS (N is A, T, G, C; S is G, C) codons at the sites corresponding to residues in positions P1, P4, P6, and P9 of each of the three 9mer core sequences chosen for mutagenesis were used for library construction and can be found in Table S2. For the first library, PCR with *Vent* DNA polymerase and pCTK-EcAII as template was carried out to generate two fragments from the primer pairs pCTKFor/3′ MSSA-NNS and 5′ MSSA-NNS/T7term, respectively. The DNA fragments obtained from these PCRs were electrophoresed and purified using a QIAGEN gel purification kit. Equimolar quantities of the two fragments were then mixed and subjected to overlap–extension PCR using the primers pCTKFor/T7term. The resulting 1.5-kb PCR product was digested with *NcoI-NotI* and ligated into pCTK-T12A digested with the same enzymes. The ligation mixture was then transformed into electrocompetent *E. coli* JC1 harboring plasmid pQE80L-GFP(11.3.3) (6), yielding ∼10^7 individual transformants. The clones were pooled and stored in 15% glycerol at −80 °C in aliquots. The second and third libraries were constructed analogously, using the internal primers 5′ INAS-NNS/3′ INAS-NNS or 5′ VQAQ-NNS/3′ VQAQ-NNS in place of 5′ MSSA-NNS/3′ MSSA-NNS, respectively.

Colorimetric Asparaginase Activity Assay. A colorimetric asparaginase assay using L-aspartic acid β-hydroxomate (AHA) (7) was used to isolate active asparaginase clones from the final FACS-sorted population of each library. Following the final round of sorting, the polyclonal gene cassette of the collected population was amplified using primers pCTKFor/T7term, digested with *NcoI-NotI*, and subcloned into pET-28a digested with the same restriction enzymes. The ligation mixture was transformed into electrocompetent *E. coli* JC2 and single colonies were used to inoculate 90 μL 2xYT supplemented with 30 μg/mL kanamycin over two 96-well plates. Following 2 h of incubation (with shaking at 350 rpm) at 37 °C, protein expression was induced by adding an additional 90 μL 2xYT (30 μg/mL kanamycin, 1 mM IPTG) to each well. After 3 h of induction at 37 °C, 120 μL from each well was transferred to a fresh 96-well plate and cells were harvested by centrifugation (3,500 × *g*, 4 C, 10 min). The cell pellets were then resuspended in 120 μL B-PER Bacterial Protein Extraction Reagent (Thermo Scientific), incubated at 25 °C for 20 min with

shaking at 350 rpm, and subsequently pelleted (3,500 × *g*, 4 C, 15 min). The resulting supernatants were transferred to a Ni^{2+}-NTA HisSorb Plate (QIAGEN) and stored at 4 °C overnight. After decanting the supernatant and rinsing twice with wash buffer (50 mM Tris-HCl, 100 mM NaCl, 25 mM imidazole, pH 8), 50 μL of 10 mM AHA in activity buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) was added to each well. Following incubation with substrate at 25 °C for 20 min, 50 μL color reagent (2% 8-hydroxyquinoline in ethanol/1 M Na_2CO_3, 1:3 by volume) was added to each well and the plate was covered, heated in a 100 °C oven for 90 s, and allowed to cool at 4 °C for 15 min. Activity within each well was quantitated by measuring the absorbance at 705 nm (Synergy HT Fluorescent Platereader, BioTek).

Expression and Purification of EcAII Variants. *E. coli* JC2 harboring pET-28a encoding either WT EcAII or an isolated mutant EcAII (p28pelHisEcAII, p28pelHis1.1.C4, p28pelHis2.2.G10, and p28pelHis3.1.E2) were cultured overnight at 37 °C in 2xYT medium supplemented with 30 μg/mL kanamycin and used to inoculate 250 mL fresh medium (1:100). When the A_{600} reached 0.5–0.7, the cells were transferred to 25 °C and allowed to equilibrate for 20 min, at which point the culture was supplemented with IPTG to a final concentration of 1 mM to induce protein expression. After 16 h of incubation at 25 °C, the cells were harvested by centrifugation at 10,000 × *g* for 10 min. The cell pellet was resuspended in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, pH 8), lysed by three passes through a French pressure cell, and subsequently pelleted at 40,000 × *g* for 45 min. The resulting supernatant (soluble fraction) was decanted, diluted 1:1 in binding buffer, and mixed with 0.5 mL of preequilibrated nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin. After incubation for 90 min at 4 °C with gentle rotation, the solution was applied to a 5 mL polypropylene column. The resin was then washed with 25 bed volumes binding buffer and 25 bed volumes wash buffer (50 mM Tris-HCl, 100 mM NaCl, 25 mM imidazole, pH 8) before the resin was incubated with 4 mL elution buffer (50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8) for 10 min and collected dropwise. The eluted fractions were concentrated using an Amicon Ultra 10 K molecular weight cutoff (MWCO) filter and purified into PBS by gel filtration on a Superdex 200 column (Amersham Pharmacia).

Determination of Kinetic Parameters. The kinetics of L-Asn hydrolysis were determined with freshly purified enzyme as described previously (8). Briefly, reactions of each asparaginase variant (10–20 nM enzyme) with L-Asn (0 to $5 \times K_M$) were carried out at 37 °C in 50 mM Tris-HCl, 100 mM NaCl (pH 7.4) in a total volume of 100 μL, and were subsequently quenched with 5 μL of 12% (wt/vol) trichloroacetic acid. An aliquot of the quenched reaction mixture was then mixed with a molar excess (relative to substrate) of *o*-phtalaldehyde (OPA) reagent and brought to a final volume of 100 μL with borate buffer. The resulting solutions were analyzed by HPLC using an Agilent ZORBAX Eclipse AAA Column (C18 reverse phase, 5 μm, 4.6 mm× 150 mm). All reactions were conducted at least in triplicate and the observed rates were fit to the Michaelis-Menten equation using Kaliedagraph (Synergy).

Asparaginase Serum Stability. Approximately 250 μg of WT EcAII or variant 3.1.E2 was mixed with 1 mL pooled human serum (Innovative Research) and incubated at 37 °C. At various time points, a 15-μL aliquot from each sample was removed and used to set up triplicate reactions in which 5 μL per aliquot was added to 50 μL 10 mM AHA in activity buffer in a microtiter plate. After allowing the reaction to proceed for 5 min at room temperature, 50 μL color reagent was added to each well, the plate was covered, heated in a 100 °C oven for 90 s, and then allowed to cool at 4 °C for 15 min. Activity within each well was quantitated by measuring the absorbance at 705 nm (Synergy HT Fluorescent Platereader, BioTek) and the average values for each enzyme-containing sample were then normalized by subtracting the average value measured for the control sample at the same time point.

Antigen Preparation and EcAII Peptide Library. Endotoxin contamination of purified enzymes was reduced by a previously described phase separation technique using the detergent Triton X-114 (9). Following six to eight phase separation cycles, protein was buffer exchanged against sterile, commercially purchased 1× PBS (Gibco) using an Amicon Ultra 10 K MWCO filter to remove any detergent that may have persisted within the solution. Enzyme preparations treated by this procedure retained normal activity. The endotoxin levels of the enzyme were determined by Limulus Amebocyte Lysate (LAL) assay and observed to be <7 endotoxin units/100 μg protein. A collection of 32 overlapping 20 mer EcAII peptides (Table S3), staggered by 10 amino acids and spanning the entire primary sequence, were synthesized by GenScript. An additional set of peptides corresponding to sequences containing the engineered mutations in 3.1.E2 were also synthesized (Abgent).

Cytokine Measurements by ELISPOT and Computer-Assisted ELISPOT Image Analysis. Cytokine ELISPOT assays were performed as described previously (10). Briefly, ELISPOT plates (Multiscreen IP, Millipore) were coated overnight with 2 μg/mL Interferon (IFN)-γ-specific capture antibody (AN-18; eBioscience) diluted in PBS. The plates were blocked with 1% BSA in PBS for 1 h at room temperature and then washed four times with PBS. Lymph node cell suspensions were plated at 5×10^5 cells/well with either whole antigen or with EcAII overlapping peptides and incubated at 37 °C for 24 h. Note that cells were plated with the whole antigen or overlapping peptides corresponding to the EcAII variant used to immunize the mouse from which they were isolated. Subsequently, the cells were removed by washing three times with PBS and four times with PBS/Tween, and IFN-γ-specific biotinylated detection Ab (R4-6A2; 0.5 μg/mL, eBioscience) was added and incubated overnight. The plate-bound secondary antibody was then incubated with streptavidin-alkaline phosphatase (Dako), and cytokine spots were visualized by 5-bromo-4-chloro-3-indolyl phosphate/NBT phosphatase substrate (KPL). Image analysis of ELISPOT assays was performed on a Series 2 Immunspot analyzer and software (Cellular Technology) as described previously. In brief, digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots based on the comparison of experimental (containing T-cells and APC with Ag or peptide) and control wells (T-cells and APC without Ag or peptide). Following the separation of spots that were touched or partially overdeveloped, nonspecific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted.

Detection of Antigen-Specific Antibody Titer by ELISA. Serum was obtained by terminal cardiac puncture from mice immunized by subcutaneous injection with either WT EcAII or 3.1.E2, as described in the main text method *Transgenic Mice*. Microtiter plates (eBioscience 44-2504-21) were coated overnight at 4 °C with 1 μg of antigen (WT EcAII or 3.1.E2) in PBS and blocked for an additional 1 h at room temperature with 1× assay diluent (eBioscience # 00-4202-56). Serial dilutions of sera were added to wells coated with the corresponding immunizing antigen and incubated for 2 h at room temperature. The plates were washed and incubated with ImmunoPure goat anti-mouse IgG conju-

gated with horseradish peroxidase for 1 h at room temperature. The plates were subsequently washed and incubated with tetramethylbenzidine substrate for 15 min at room temperature before the reactions were stopped by addition of 2 M H_2SO_4. The absorbance was read (450 nm) using an ELISA microplate reader (μQuant; Biotek Instruments). End-point titers were calculated by using an absorbance corresponding to a control well (PBS substituted for sera) as the cutoff value.

1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.
2. Sturniolo T, et al. (1999) Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol* 17:555–561.
3. Singh H, Raghava GP (2001) ProPred: Prediction of HLA-DR binding sites. *Bioinformatics* 17:1236–1237.
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5. Southwood S, et al. (1998) Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* 160:3363–3373.
6. Yoo TH, Link AJ, Tirrell DA (2007) Evolution of a fluorinated green fluorescent protein. *Proc Natl Acad Sci USA* 104:13887–13890.
7. Wehner A, et al. (1992) Site-specific mutagenesis of Escherichia coli asparaginase II. None of the three histidine residues is required for catalysis. *Eur J Biochem* 208:475–480.
8. Cantor JR, Stone EM, Chantranupong L, Georgiou G (2009) The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with beta-aspartyl peptidase activity. *Biochemistry* 48:11026–11031.
9. Aida Y, Pabst MJ (1990) Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J Immunol Methods* 132:191–195.
10. Forsthuber TG, et al. (2001) T cell epitopes of human myelin oligodendrocyte glycoprotein identified in HLA-DR4 (DRB1*0401) transgenic mice are encephalitogenic and are presented by human B cells. *J Immunol* 167:7119–7125.

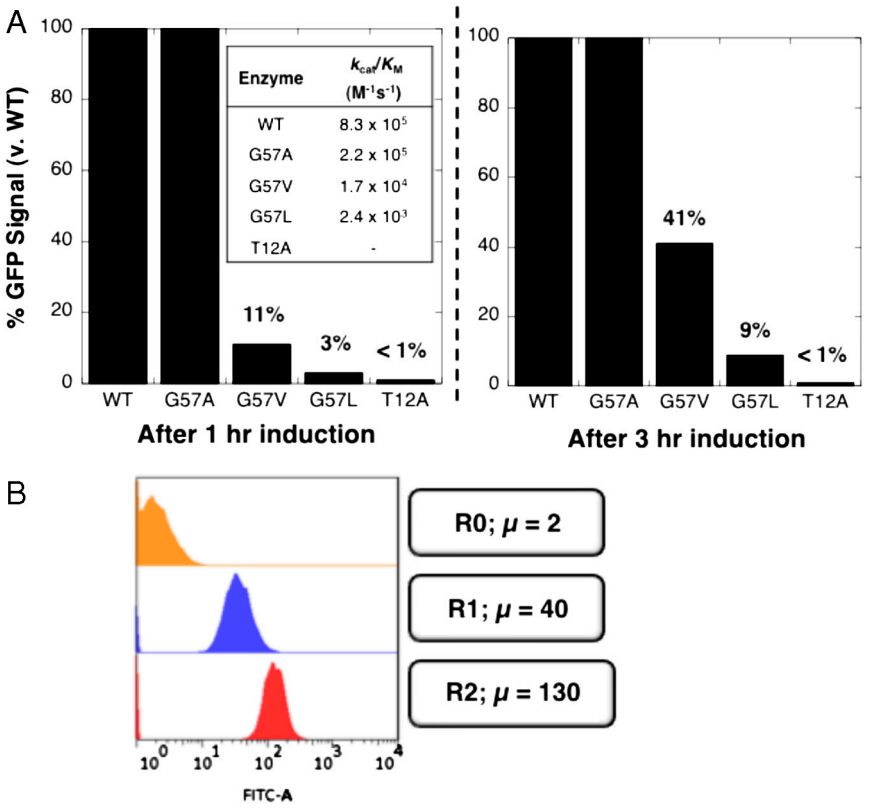

Fig. S1. Validation of the neutral drift screen for cells expressing EcAII variants with high catalytic activity. (*A*) Relative GFP signal of a panel of *E. coli* JC1 cells expressing EcAII variants with different catalytic efficiencies for the hydrolysis of the L-Asn analog AHA (1). The T12A mutant displays no AHA hydrolysis activity above background in this assay. (*B*) Fluorescence histograms showing three-round enrichment of JC1 cells expressing EcAII from a mixture containing a 1:10,000 excess of JC1 cells expressing EcAII-T12A. After three rounds of sorting, DNA sequencing revealed that five of eight clones selected at random encoded EcAII. μ, geometric mean fluorescence.

1. Derst C, Henseling J, Röhm KH (2000) Engineering the substrate specificity of Escherichia coli asparaginase. II. Selective reduction of glutaminase activity by amino acid replacements at position 248. *Protein Sci* 9:2009–2017.

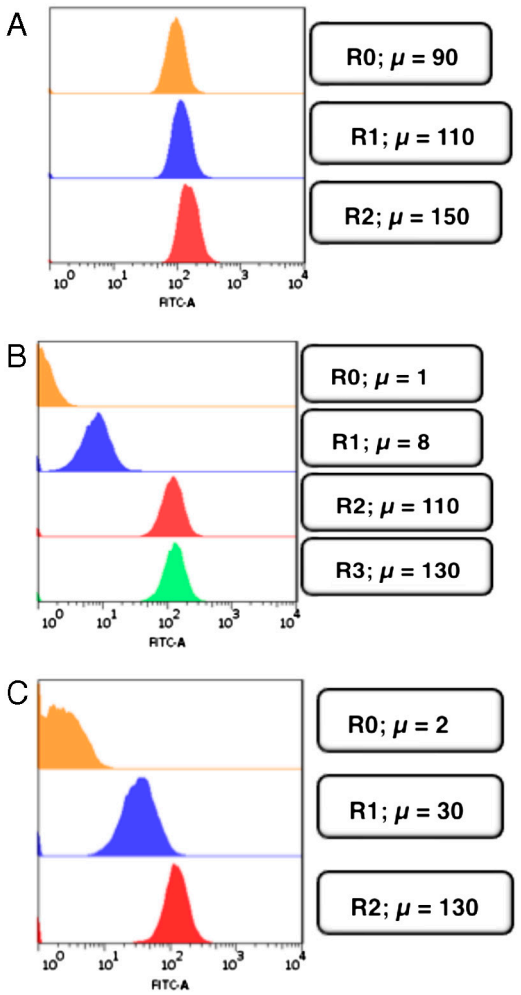

Fig. S2. FACS histograms of four residue saturation libraries at the anchor positions in the predicted T-cell epitope 9-mer peptides M_{115}, I_{216}, V_{304} (see main text for nomenclature) by the neutral drift assay. Each library comprised of $>10^7$ transformants generated by randomizing the P1, P4, P6 and P9 positions of the respective T-cell epitopes using the NNS scheme. (*A*) M_{115} library, (*B*) I_{216} library, and (*C*) V_{304} library. μ, geometric mean fluorescence.

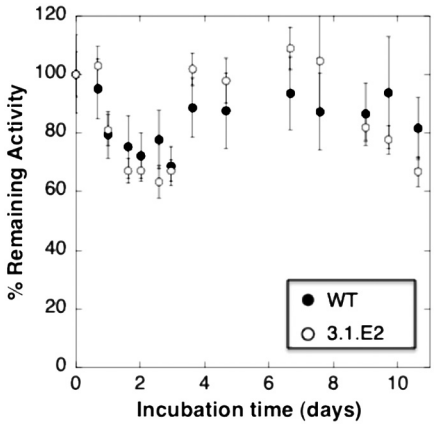

Fig. S3. In serum stability of WT EcAII and 3.1.E2 measured quantitatively by percent remaining activity over time. Error bars shown are for SD.

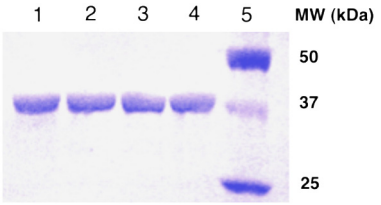

Fig. S4. Reducing SDS-PAGE showing the purity of purified WT EcAII and engineered EcAII variants. Lane 1, WT EcAII; lane 2, 1.1.C4; lane 3, 2.2.G10; lane 4, 3.1.E2; lane 5, molecular weight (MW) standards.

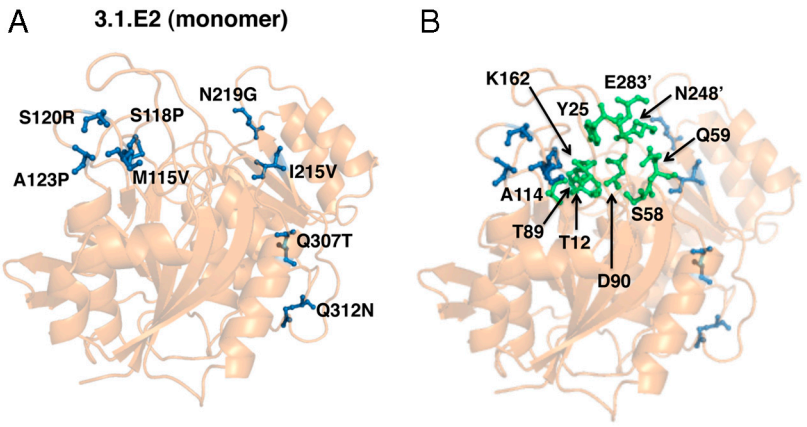

Fig. S5. Location of amino acid mutations of 3.1.E2 in relation to the active site residues of EcAII. (*A*) Location of the eight amino acid mutations differentiating wild type EcAII and 3.1.E2. (*B*) Active site residues of EcAII (1) are depicted in green and numbered. Residues numbered with a prime are located in an adjacent monomer.

1. Sanches M, Krauchenco S, Polikarpov I (2007) Structure, substrate complexation and reaction mechanism of bacterial asparaginases. *Curr Chem Biol* 1:75–86.

Table S1. Plasmids used in this study

Plasmids	Relevant characteristics	Reference or source
pET-28a	Kan^R, T7 promoter	Novagen
pHisEcAII	Kan^R, T7 promoter, encodes His6x-EcAII	This study
pET-26b	Kan^R, T7 promoter	Novagen
pPelbHisEcAII	Kan^R, T7 promoter, encodes sspelb-His6x-EcAII	This study
pPelbHisT12A	Kan^R, T7 promoter, encodes sspelb-His6x-EcAII(T12A)	This study
pCTK	Kan^R, *tet* promoter, CloDF ori	This study
pASK75	Amp^R, *tet* promoter	(1)
pCDF-1b	Sp^R, T7 promoter, CloDF ori	Novagen
pCTK-EcAII	encodes sspelb-His6x-EcAII in pCTK	This study
pCTK-T12A	encodes sspelb-His6x-EcAII(T12A) in pCTK	This study
pCTK-G57A	encodes sspelb-His6x-EcAII(G57A) in pCTK	This study
pCTK-G57V	encodes sspelb-His6x-EcAII(G57V) in pCTK	This study
pCTK-G57L	encodes sspelb-His6x-EcAII(G57L) in pCTK	This study
p28pelHisEcAII	encodes sspelb-His6x-EcAII in pET-28a	This study
p28pelHis1.1.C4	encodes sspelb-His6x-1.1.C4 in pET-28a	This study
p28pelHis2.2.G10	encodes sspelb-His6x-2.2.G10 in pET-28a	This study
p28pelHis3.3.E2	encodes sspelb-His6x-3.1.E2 in pET-28a	This study
pQE80L-GFP(11.3.3)	Amp^R, lac promoter, encodes GFP(11.3.3)	(2)

1. Skerra A (1994) Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in Escherichia coli. *Gene* 151:131–135.
2. Yoo TH, Link AJ, Tirrell DA (2007) Evolution of a fluorinated green fluorescent protein. *Proc Natl Acad Sci USA* 104:13887–13890.

Table S2. Primers used in this study

Primer name	Nucleotide sequence (5′ → 3′)
ansBFor	GTGCAGCACATATGTTACCCAATATCACCA
ansBRev	GGCGGGATCCTTAGTACTGATTGAAGA
T12AFor	GTGCAGCACATATGTTACCCAATATCACCATTTTAGCAACCGGCGGGGCCA
T7term	GCTAGTTATTGCTCAGCGG
tetFor	CTAGCTAGTCTAGAGCGGAGCCTATGGAAAAACGC
tetRev	CATGCCATGGCACTTTTCTCTATCACTGATAGGG
KanFor	GGGCCGGCAGGCGCTCCATTGCCCAGTCGGCTAAGGGATTTTGGTCATG
KanRev	GCGTAGCGACCGAGTGAGCTAGCTTATTAGAAAAACTCATCGAGCATC
5′ ansA KO	CCTCACGTATATACTTTTGCTCTTTCGATATCATTCATATCAATATCATGATTCCGGGGATCCGTCGACC
3′ ansA KO	ACAGGGCGCGAGGGGGCATTACAGTCTCCTTAATCATCCGGCGTCAGTTCTGTAGGCTGGAGCTGCTTCG
5′ ansB KO	CAGAGCTAAGGGATAATGCGTAGCGTTCACGTAACTGGAGGAATGAAATGATTCCGGGGATCCGTCGACC
3′ ansB KO	AGCCCCGGCACGATACCGGGGCGAGGCGATTAGTACTGATTGAAGATCTGTGTAGGCTGGAGCTGCTTCG
5′ iaaA KO	TGATATTTATAGCAAAAGTGGCGAACCACCCTTAATGGACGAATACTATGATTCCGGGGATCCGTCGACC
3′ iaaA KO	CCGCCAGCACATTACCGGCATCAAGTTCATCACTGTGTGGCAACGGTGTCTGTAGGCTGGAGCTGCTTCG
5′ aspC KO	TACCCTGATAGCGGACTTCCCTTCTGTAACCATAATGGAACCTCGTCATGATTCCGGGGATCCGTCGACC
3′ aspC KO	TTTTCAGCGGGCTTCATTGTTTTTAATGCTTACAGCACTGCCACAATCGCTGTAGGCTGGAGCTGCTTCG
5′ tyrB KO	GTTTATTGTGTTTTAACCACCTGCCCGTAAACCTGGAGAACCATCGCGTGATTCCGGGGATCCGTCGACC
3′ tyrB KO	GCTGGGTAGCTCCAGCCTGCTTTCCTGCATTACATCACCGCAGCAAACGCTGTAGGCTGGAGCTGCTTCG
5′ MSSA-NNS	GGTCGGCGCANNSCGTCCGNNSACGNNSATGAGCNNSGACGGTCCATTCAACCTG
3′ MSSA-NNS	CAGGTTGAATGGACCGTCSNNGCTCATSNNCGTSNNCGGACGSNNTGCGCCGACC
5′ INAS-NNS	CTGCCGAAAGTCGGCNNSGTTTATNNSTACNNSAACGCANNSGATCTTCCGGCTAAAGCACTG
3′ INAS-NNS	CAGTGCTTTAGCCGGAAGATCSNNTGCGTTSNNGTASNNATAAACSNNGCCGACTTTCGGCAG
5′ VQAQ-NNS	CCGCAAAAAGCGCGCNNSCTGCTGNNSCTGNNSCTGACGNNSACCAAAGATCCGCAGCAG
3′ VQAQ-NNS	CTGCTGCGGATCTTTGGTSNNCGTCAGSNNCAGSNNCAGCAGSNNGCGCGCTTTTTGCGG
pCTKFor	CGATCAAACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGC
5′ G57A	GGTAGTGAATATCGCGTCCCAGGACATGAACG
5′ G57V	GGTAGTGAATATCGTGTCCCAGGACATGAACG
5′ G57L	GGTAGTGAATATCCTGTCCCAGGACATGAACG
3′ G57mp	GGTGACGTCACGGCCATCAAGCACGGTGTCATTCATC

Table S3. Overlapping synthetic peptides of WT EcAII for T-cell activation assays

Peptide name	Peptide sequence
WTp1-20	MLPNITILATGGTIAGGGDS
WTp11-30	GGTIAGGGDSATKSNYTVGK
WTp21-40	ATKSNYTVGKVGVENLVNAV
WTp31-50	VGVENLVNAVPQLKDIANVK
WTp41-60	PQLKDIANVKGEQVVNIGSQ
WTp51-70	GEQVVNIGSQDMNDNVWLTL
WTp61-80	DMNDNVWLTLAKKINTDCDK
WTp71-90	AKKINTDCDKTDGFVITHGT
WTp81-100	TDGFVITHGTDTMEETAYFL
WTp91-110	DTMEETAYFLDLTVKCDKPV
WTp101-120	DLTVKCDKPVVMVGAMRPST
WTp111-130	VMVGAMRPSTSMSADGPFNL
WTp121-140	SMSADGPFNLYNAVVTAADK
WTp131-150	YNAVVTAADKASANRGVLVV
WTp141-160	ASANRGVLVVMNDTVLDGRD
WTp151-170	MNDTVLDGRDVTKTNTTDVA
WTp161-180	VTKTNTTDVATFKSVNYGPL
WTp171-190	TFKSVNYGPLGYIHNGKIDY
WTp181-200	GYIHNGKIDYQRTPARKHTS
WTp191-210	QRTPARKHTSDTPFDVSKLN
WTp201-220	DTPFDVSKLNELPKVGIVYN
WTp211-230	ELPKVGIVYNYANASDLPAK
WTp221-240	YANASDLPAKALVDAGYDGI
WTp231-250	ALVDAGYDGIVSAGVGNGNL
WTp241-260	VSAGVGNGNLYKSVFDTLAT
WTp251-270	YKSVFDTLATAAKTGTAVVR
WTp261-280	AAKTGTAVVRSSRVPTGATT
WTp271-290	SSRVPTGATTQDAEVDDAKY
WTp281-300	QDAEVDDAKYGFVASGTLNP
WTp291-310	GFVASGTLNPQKARVLLQLA
WTp301-320	QKARVLLQLALTQTKDPQQI
WTp311-326	LTQTKDPQQIQQIFNQY