The structure of SpoT reveals evolutionary tuning of enzymatic output through constraint of the conformational landscape

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Online Methods

Multiple sequence alignment

Sequences were aligned with MAFFT v7.164b with the L-ins-i strategy [49], and alignments were visualised with Jalview [50].

Construction of bacterial strains and plasmids

The strains and plasmids used in this study are listed in supplementary **Supplemental Data**. To construct the plasmids for the expression of *A. baumanii* RelA and SpoT, the corresponding *relA* and *spoT* genes were amplified with Q5 polymerase (NEB) from *A. baumanii* (AB19606) cells using primer pairs (**Supplemental Data** harbouring restriction enzyme cleavage sites *Bmt*I (upstream oligo) and *Eco*RI (downstream oligo). And the backbone of the pET28b plasmid was amplified using oligos R-pET-hisTEV-Bmt and F-pET-synt. All three PCR products were cleaved *Bmt*I and *Eco*RI enzymes (NEB) at 37 °C for 1 hour, the plasmid backbone dephosphorylated with rSAP (NEB) for 30 minutes. The gene products were ligated (ligase NEB) into the plasmid backbone at 20 °C, overnight and the mixture transformed into *E. coli* MC1061 strain. The resulting plasmids verified by sequencing (Eurofins).

To construct the plasmids for the expression of the SpoT$_{Ab}$ mutated proteins (pET28b-SpoT$_{Ab}$*) the deletions and point mutations to the *spoT*$_{\mathrm{Ab}}$ gene in the plasmid pET28b-SpoT$_{Ab}$ were introduced by amplifying the entire pET28b-SpoT$_{Ab}$ plasmid with Q5 polymerase (NEB) using primer-pairs (listed in **Supplemental Data**) of which one of the primers harboured the desired sequence for the amino-acid substitution to introduce the mutation. For constructing plasmids to express catalytic domain of SpoT$_{Ab}$ or RelA$_{Ab}$ (SpoT$_{Ab}^{\mathrm{NTD}}$ or RelA$_{Ab}^{\mathrm{NTD}}$) the primers harboured a stop codon and introduced that to the desired position. For constructing the deletions of other domains of SpoT$_{Ab}$, the used primers were flanking the region to be deleted. In all cases, the acquired product was treated with *Dpn*I (NEB) to remove the template plasmid, purified through a PCR purification column (Omega), phosphorylated with PNK (NEB) and ligated (NEB). The mixture was then transformed into *E. coli* MC1061 strain and resulting plasmids verified by sequencing (Genewiz, Eurofins).

To construct the plasmid pHR1186, an apramycin cassette was amplified from pMHL2 by PCR with the primers 2213/2214 (**Supplemental Data**). The PCR product was then digested with *Nco*I/*Bgl*II and ligated into the pK18mobsacB vector digested with the same enzymes.

To construct pHR1187 and pHR1188, upstream and downstream regions of *A. baumannii relA* (ABUW_3302) or *spoT* (ABUW_0309) were amplified by PCR with the

primers 2133/2134 and 2135/2136 or 2137/2138 and 2139/2140 (**Supplemental Data**) respectively. The PCR products were then digested with *Xho*I/*Eco*RI (upstream region) and *Eco*RI/*Nhe*I (downstream region), and ligated into the pTOX5-Tet vector cut with *Xho*I and *Nhe*I. Then, fused upstream and downstream regions were PCR amplified with primers 2110/2188 (**Supplemental Data**) and ligated into the pHR1186 digested with *Sma*I.

To construct pHR1467, the downstream region of *spoT* was PCR amplified with primers 2139/2140. The PCR product was digested with *Xba*I/*Nhe*I and ligated into pH1186 digested with the same enzymes.

To generate pHR1440, pHR1441, pHR1442, pHR1443, pHR1444 and pHR1445 a part of *spoT* CDS (ABUW_0309) was PCR amplified with the primers 2874/2875, 2876/2877, 2878/2879, 2880/2881, 2882/2883 and 2884/2885 (**Supplemental Data**), respectively and *Xba*I digested. The PCR products were then ligated into pHR1467 digested with *Sma*I/*Xba*I.

To generate pHR1446, pUC18-mini-Tn7-LAC backbone was PCR amplified with primers 2856/2857 and digested with *Avr*II. An *Avr*II/*Eco*RV fragment from pRCG-eGFP containing an apramycin cassette was then ligated to the previously *Avr*II digested PCR fragment.

To construct pHR1447, a part of pHR1446 was PCR amplified with the primers 2906/2907, digested with *Ase*I/*Sca*I and ligated into pHR1446 digested with *Nde*I/*Sca*I. A part of the intermediate plasmid was PCR amplified with the primers 2464/2908, digested with *Avr*II/*Bam*HI and ligated into the same plasmid digested with *Avr*II/*Bam*HI.

To generate pHR1448, pHR1447 backbone without its origin of replication was PCR amplified with primers 3100/3101 and digested with *Sca*I. The pBAD33 origin of replication was PCR-amplified with the primers 3098/3099 and ligated to the previously ScaI digested PCR fragment.

To generate pHR1449, *A. baumannii relA* (ABUW_3302) CDS was PCR amplified with the primers 2867/2868 and digested with *Bam*HI/*Pst*I. The digested PCR product was ligated into pHR1446 digested with the same enzymes.

To generate pHR1450 to pHR1459, *A. baumannii spoT* CDS were PCR amplified with the primers 2909/2910 on the corresponding pET28b plasmids and digested with *Nde*I/*Eco*RI. The digested PCR products were ligated into pH1447 digested with the same enzymes.

To construct pHR1460, pAGS1 backbone was PCR amplified with the primers 2417/2418 and digested with *Eco*RI. An *Eco*RI fragment from pRCG-eGFP containing an apramycin cassette was then ligated to the previously *Eco*RI digested PCR fragment. To generate pHR1461, *A. baumannii relA* (ABUW_3302) locus was amplified by PCR with the

primers 2472/2473, digested with *Avr*II/*Bgl*II and ligated into pHR1460 digested with the same enzymes. To generate pHR1462, a PCR fragment containing a tetracycline resistance cassette was PCR-amplified with the primer 3000/3001, digested with *Avr*II/*Xho*I and ligated to pHR1461 digested with the same enzymes.

To generate the *A. baumannii* Δ*relA* strain, pHR1187 was introduced in the WT strain by natural transformation. Apramycin-resistant and sucrose-sensitive recombinant clones were first selected to launch an overnight culture in LB medium. In a second step, apramycin-sensitive and sucrose-resistant clones were screened by PCR using the primers 2141/2142 to identify Δ*relA* recombinant.

For the *A. baumannii* Δ*relA* Δ*spoT* strain the same protocol was used by introducing the pHR1188 into the Δ*relA* strain, recombinants were screened with the primers 2143/2144. pHR1440, pHR1441, pHR1442, pHR1443, pHR1444, pHR145 were introduced in the *A. baumannii* WT or Δ*relA* strains to generate *spoT1-454, spoT1-560,* Δ*relA-spoT1-196,* Δ*relA-spoT1-339,* Δ*relA-spoT1-385,* Δ*relA-spoT1-454,* Δ*relA-spoT1-560,* Δ*relA-spoT1-641* strains using the same protocol, recombinants were screened with the primers 2143/2144.

To complement strains with the P*tac*::*relA* construct, pHR1449 and pTNS2 were co-transformed by natural transformation. To complement strains with the P*tac*::*spoT* construct and allelic variants, pHR1450 to pHR1459 and pTNS2 were co-transformed by natural transformation. Insertion downstream *glmS* at the attTn7 site was screened by PCR with the primers 2422/2466 on apramycin-resistant clones. To complement strains with the P*relA*::*relA* construct pHR1461 or pHR1462 were introduced by natural transformation.

Growth assays

E. coli BW25113 cells were transformed with expression constructs either based on a high-copy IPTG-inducible vector pUC derivative pMG25 (pMG25::*relA* [51], pNDM220::$relA^{\Delta RRM}$, pNDM220::*spoR* or pNDM220::$spoT^{\Delta RRM}$) or on a low-copy IPTG-inducible vector, mini R1 plasmid pNDM220 which is present in one to two copies per chromosome [52]. For solid medium growth assays, ten-fold serial dilutions of overnight LB cultures were spotted onto LB agar supplemented with 30 µg/mL ampicillin and 1 mM IPTG. For liquid medium growth assays, thousand-fold dilutions of the overnight LB cultures were made in liquid LB supplemented with 30 µg/mL ampicillin and 1 mM IPTG, seeded on a 100-well honeycomb plate (Oy Growth Curves AB Ltd, Helsinki, Finland), and plates incubated in a Bioscreen C (Labsystems, Helsinki, Finland) at 37 °C with continuous medium shaking. Growth rates (μ_2) were calculated as slopes of linear regression lines through $\log_2$-transformed OD_{600} data points.

Virulence assays in G. mellonella

G. mellonella larvae (TruLarv) were purchased from Biosystems Technology (United Kingdom). Upon reception larvae were stored at 17 °C until further experiment. The day of the inoculation, larvae were incubated 1h at 4 °C before injection. Overnight culture of *A. baumannii* were washed twice and diluted to a cell density of ~ 2 x 10^7 CFU/mL in 0.9 % NaCl, 10 µL were injected in the bottom left proleg of each larva. Eight larvae were inoculated per strain, and incubated at 37 °C in the dark. Viability of the larvae was scored every 24 h for 6 days.

Protein purification

For the production all the *A. baumanii* proteins, the pET28b plasmids with the expression constructs for the desired proteins were transformed to BL21 (DE3) *E. coli* cells. The bacteria harbouring these plasmids were grown overnight at 37 °C in LB media supplemented with kanamycin 50 µg/mL, then diluted 500 times to fresh LB media (with kanamycin) and grown at 37 °C up to an OD_{600} 0.6. The temperature was lowered to 25 °C and protein expression induced with 0.5 mM IPTG. The proteins were expressed at least 5 h and cells harvested by centrifugation. The cells were resuspended in buffer (50 mM Tris pH 8, 1 M KCl, 2 mM $MgCl_2$, 1 mM TCEP, 0.002% mellitic acid) supplemented with cOmplete protease inhibitor cocktail (Roche), flash-frozen and stored at -80 °C until purification. Before cracking the cells with a French press, DNase I treatment was carried out (0.25 mg per cells from 1 L of culture). The supernatant was separated from the pellet by centrifugation and loaded on to a Cobalt gravity flow column previously equilibrated with Buffer A (50 mM HEPES pH 8, 500 mM NaCl, 500 mM KCl, 10 mM $MgCl_2$, 1 mM TCEP, 0.002% mellitic acid). The column was washed with 10 column volumes of Buffer A and the protein was eluted with 4 column volumes of Buffer B, (buffer A containing 200 mM imidazole). To remove any remaining contaminants and imidazole, the elution fraction was immediately transferred to a size exclusion chromatography (SEC) column (GE Healthcare), previously equilibrated in the SEC buffer [50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM KCl, 2 mM $MgCl_2$, 1 mM TCEP, 0.002% mellitic acid (and 1 mM $MnCl_2$ for all SpoT proteins)]. The fractions containing the protein were concentrated to around 1 mg/mL and the His-tag cleaved with TEV protease (1:100 molar ratio) at room temperature overnight. The cleaved protein was purified from the mixture by loading it on the SEC column (equilibrated in the SEC buffer if not stated otherwise) or (in the case of $SpoT_{Ab}^{NTD}$ $SpoT_{Ab}^{1-195}$, $RelA_{Ab}$, and $RelA_{Ab}^{NTD}$) the mixture was loaded on a Cobalt gravity flow column previously

equilibrated in the SEC buffer, and the flow-through collected. The proteins were prepared immediately for experiments or flash frozen in liquid nitrogen and stored at –80 °C. Rel_{Bs} was purified as in [53].

Preparation of ^{3}H-labeled ppGpp

250 nM *E. faecalis* RelQ [54] was incubated in reaction buffer (10 mM $MgCl_2$, 20 mM Tris-HCl pH 8.0, 100 mM NaCl) together with 2 mM ATP and 1 mM ^{3}H-GDP (specific activity 80 cpm/pmol, PerkinElmer) for 2 h at 37 °C to produce ^{3}H-ppGpp. The resultant mixture was loaded on strong anion-exchange column (MonoQ 5/50 GL; GE Healthcare), and nucleotides were resolved by a 0.5–2,000 mM LiCl gradient (with 0.5 mM EDTA, 2.5 mM Tris-HCl pH 8.0). Peak fractions containing ^{3}H-ppGpp were pooled and precipitated by addition of potassium acetate to a final concentration of 0.3 M followed by addition of 2.5 volumes of ethanol. The suspension was incubated at –20 °C overnight and centrifuged (14,800 rpm, 30 min, 4 °C). The resulting pellets were washed with absolute ethanol, dried, dissolved in 10 mM HEPES-KOH buffer (pH 8.2) and stored at –80 °C.

Crystallisation

Prior to crystallization $SpoT_{Ab}$ and $SpoT_{Ab}^{NTD}$ were purified from the TEV-cleavage reaction in the GF buffer and concentrated to 8-10 mg/mL. Before crystallization, $SpoT_{Ab}$ was supplemented with $MnCl_2$ (1 mM) and incubated for 10 min at room temperature. The screening of crystallization conditions was carried out using the sitting-drop vapor-diffusion method. The drops were set up in Swiss (MRC) 96-well two-drop UVP sitting-drop plates using the Mosquito HTS system (TTP Labtech). Drops of 0.1 µL protein and 0.1 µL precipitant solution were equilibrated to 80 µL precipitant solution in the reservoir. Commercially available screens LMB and SG1 (Molecular Dimensions) were used to test crystallization conditions. The conditions resulting in protein crystals (SG1, position G6 for $SpoT_{Ab}^{NTD}$ and LMB screen position H7 for $SpoT_{Ab}$) were repeated as 2 µL drops. Crystals were harvested using suitable cryo-protecting solutions and an additional 20 mM of ppGpp (Jena Bioscience) was added to the cryo-protecting solution of $SpoT_{Ab}$ to prevent off-binding and vitrified in liquid N_2 for transport and storage before X-ray exposure. X-ray diffraction data was collected at the SOLEIL synchrotron (Gif-sur-Yvette, Paris, France) on the Proxima 1 (PX1) and Proxima 2A (PX2A) beamlines using an Eiger-X 16M detector. Because of the high anisotropic nature of the data from all the crystals we performed anisotropic cutoff and correction of the merged intensity data as implemented on the STARANISO server (http://staraniso.globalphasing.org/)

using the DEBYE and STARANISO programs. In the case of the crystals of the SpoT$_{Ab}$-ppGpp complex, the analysis of the data suggested a resolution of 2.51 Å (with 3.29 Å in *a**, 2.75 Å in *b** and 2.51 Å in *c**). For Mn^{2+}-free SpoT$_{Ab}^{NTD}$, the analysis of the data suggested a resolution of 2.79 Å (with 2.94 Å in *a**, 2.94 Å in *b** and 2.79 Å in *c**).

Structure determination

The data were processed with the XDS suite [55] and scaled with XSCALE or Aimless. In all cases, the unit-cell content was estimated with the program MATTHEW COEF from the CCP4 program suite [56]. Molecular replacement was performed with Phaser [57]. The crystals of the SpoT$_{Ab}$-ppGpp complex diffracted on average to ≈2.9 Å. We used the coordinates of Rel$_{Tt}^{NTD}$ as search model for the NTD (PDBID 6S2T) [58] and the coordinates of *E. coli* RelA for the CTD [59-61]. Because of the intrinsic interdomain dynamics of Rel catalytic domains we search for an MR solution using each catalytic domain as an independent ensemble. The MR solution from Phaser was used in combination with combined with Rosetta as implemented in the MR-Rosetta [62] suit from the Phenix package [63]. After several iterations of manual building with Coot [64] and maximum likelihood refinement as implemented in Buster/TNT [65], the model was extended to cover all the residues (R/Rfree of 21.7/25.0 %).

In the case of metal-free SpoT$_{Ab}^{NTD}$, we used the coordinates of the individual HD and SYN domains from the structure of SpoT$_{Ab}$ as search model for MR in Phaser. The structure was completed after several iterations of manual building with Coot [64] and maximum likelihood refinement as implemented in Buster/ Buster/TNT [65] to an R/Rfree of 24.6/28.6 %. In every case the geometrical restraints of all small molecules were generated with the Grade Web Server (http://grade.globalphasing.org). **Table S1** details all the X-ray data collection and refinement statistics.

Analytical size exclusion chromatography (SEC)

For the analytical size exclusion chromatography (SEC) 100 µL of each protein at a concentration of 1 mg/mL was loaded on a Superdex 200 Increase 10300 column (GE Healthcare) previously equilibrated in the SEC buffer. The progress of the chromatography was monitored by the OD_{280}.

^{3}H-ppGpp hydrolysis assay

Mutational analysis of *A. baumannii* SpoT was carried out with reaction mixtures contained 50–400 nM SpoT, 200 µM ^{3}H-ppGpp, 1 mM $MnCl_2$, in HEPES:Polymix buffer, pH 7.5 [53] (5

mM Mg^{2+} final concentration). After preincubation at 37 °C for 2 min, the reaction was started by the addition of prewarmed SpoT and 5 µL aliquots were taken throughout the time course of the reaction and quenched with 4 µL 30% formic acid supplemented with a cold nucleotide standard (4 mM GDP) for UV-shadowing. Individual quenched timepoints were spotted PEI-TLC plates (Macherey-Nagel) and nucleotides were resolved in 0.5 M KH_2PO_4 pH 3.5 buffer. The TLC plates were dried, cut into sections as guided by UV-shadowing, and ^3H radioactivity was quantified by scintillation counting in EcoLite Liquid Scintillation Cocktail (MP Biomedicals).

SpoT$_{Ab}$ Michaelis constant (K_M) and maximum reaction velocity (V_{max}) of ^3H-ppGpp hydrolysis were determined with 25-200 nM of SpoT$_{Ab}$ and 10-600 µM ^3H-ppGpp as a substrate in HEPES:Polymix buffer pH 7.5 (1 mM $MnCl_2$, 5 mM Mg^{2+} final concentration). Reaction was started by adding prewarmed SpoT$_{Ab}$. To test inhibition of SpoT$_{Ab}$ (50 nM) [3] of H-ppGpp hydrolysis (100 µM) we used 0.5-4 µM of *E. coli* ribosomes (70S), acetylated *E. coli* Val-$tRNA^{Val}$ and deacetylated *E. coli* $tRNA^{Val}$ (Chemical Block). The reaction was started by adding prewarmed SpoT$_{Ab}$ to the substrate in HEPES:Polymix buffer. The effect of manganese and EDTA on SpoT$_{Ab}$ was tested with 50-2000 µM of $MnCl_2$ or 1 mM EDTA at the substrate concentration 100 µM of ^3H-ppGpp. Reaction mixture in HEPES:Polymix buffer, pH 7.5 (5 mM Mg^{2+} final concentration) including EDTA or $MnCl_2$ and 50-400 nM SpoT$_{Ab}$ was preincubated at 37 °C for 2 min and the reaction was started by adding 100 µM (final concentration) of ^3H-ppGpp. 5 µL aliquots were taken throughout the time course and ^3H radioactivity was quantified as described above.

Chemical denaturation assays

For chemical denaturation assays the proteins were transferred to a denaturation (DN) buffer (25 mM Tris pH 7.5, 500 mM NaCl, 500 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM TCEP 0.002% mellitic acid) after the cleavage of the tag, flash frozen at 1 mg/mL concentration and stored at -80 °C. To measure denaturation, samples of proteins in DN buffer containing different concentration of guanidinium hydrochloride (GdHCl) were prepared. The final mixtures contained 0.2-0.3 mg/mL of protein and 0-5.4 M GdHCl. Denaturation was followed by recording tryptophan fluorescence emission spectra in a PTI fluorometer (Photon Technologies International). The samples were excited at 280 nm and denaturation was assayed by the ratio of fluorescence intensities at 355 and 335 nm. Samples were prepared in triplicate, incubated and measured at 37 °C. All data were analysed with the CDpal package in "chemical denaturation" mode [66].

Small angle X-ray scattering (SAXS)

For Small-angle X-ray scattering (SAXS) experiments the proteins were concentrated to around 8 mg/mL immediately after purification (in SEC buffer), flash frozen and stored at -80 °C. SAXS data were collected at the SWING beamlines (Soleil and ESRF synchrotrons, France) on a Pilatus 2M detector using the standard beamline setup in SEC (size exclusion) mode. The samples were prepared in 500 mM NaCl, 2 mM TCEP and 30 mM Tris-HCl pH 7.0. Frames showing radiation damage were removed before data analysis. The data were analysed with the ATSAS suite. For the SEC-SAXS we used a Shodex KW404–4F column coupled to an HPLC system, in front of the SAXS data collection capillary, to separate the excess non-complexed material and thus remove this source of background. The sample was passed at a flow rate of 0.2 ml/min and the data collected at 10 °C. *R*g values were obtained from the Guinier approximation and the *I*0 (scattering intensity at 0 concentration by extrapolation to $q = 0$, as implemented in ATSAS.

SAXS-based models were derived from the coordinates of the SpoT$_{Ab}$ in complex with ppGpp. The coordinates of the initial model were completed to account for missing loops or point substitutions using Modeller. The calculation of *ab initio* shapes based on the scattering data was done with the program DAMMIF from the ATSAS package. **Table S2** shows all the SAXS-derived parameters.

In order to model the structures of SpoTAb in the τ- and relaxed states in solution, we used the program Dadimodo [67] that refines multidomain protein structures against experimental SAXS data. The program cyclically samples Phi, and Psi rotations in the backbone of the interdomain regions defined by the user following an stochastic optimization algorithm. All the structures generated by the program are physically reliable. We used Dadimodo via the webserver application (https://dadimodo.synchrotron-soleil.fr) and defined the following rigid domains: body_1 1 – 107, 129 - 189, 801-801; body_2 201 - 239, 257 - 293, 307 – 333; body_3 380 – 443; body_4 455 – 512; body_5 572 – 605, 901-901, body_6 618 – 689 based on the linker regions observed in the structure of SpoT$_{Ab}$.

Isothermal titration calorimetry (ITC)

All titrations were performed with an affinity ITC machine (TA instruments) at 15 °C. Stock solutions of GDP (Sigma Aldrich) of 650-670 mM were diluted in the protein buffer (50 mM HEPES pH 7.5; 500 mM KCl; 500 mM; NaCl; 10 mM $MgCl_2$; 1 mM TCEP; 0.002 % mellitic acid) to a final concentration 1.0 mM. The purified SpoT$_{Ab}^{NTD}$ was concentrated by

ultrafiltration (Amicon ultra, 0.5 mL 30 kDa, Merck Millipore) to 80-100 μM. All final concentrations were verified by the absorption using a Nanodrop One (Thermo Scientific). All ITC measurements were performed by titrating 2 μL of the nucleotide into the protein using a constant stirring rate of 75 rpm. All data were processed and analysed using the NanoAnalyse and Origin software packages.

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