SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Plasmid pPB1 encodes the AAA+ domain of *E. coli* PspF, called PspF1-275 with an amino terminal 6-His tag in pET28b+ (Bordes et al., 2003). Variants of PspF1-275 were generated to yield variants: pNJ and pNZ (Supplemental Table 1S), verified by DNA sequencing.

Protein purification

PspF1-275 proteins were purified (Joly et al., 2006). σ54 and 32P-end labelling HMK (heart muscle kinase)-σ54 was purified and labelled (Cannon et al., 2000; Wigneshweraraj et al., 2003). *E. coli* core RNAP enzyme was purchased from Epicentre. Protein concentration was estimated (Lowry et al., 1951).

ATPase activity

Steady-state ATPase assays were performed at 37°C in the presence of a NADH-coupled regeneration system (Norby, 1988) in a 100 µl final volume, in buffer containing final concentrations of: 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl2, 1 mM DTT, 1 mM NADH, 10 mM Phosphoenol pyruvate, 10 U/ml Pyruvate Kinase, 20 U/ml Lactate dehydrogenase, ATP (from none to 50 mM) and PspF1-275 (from 0 to 20 µM). Mixing experiments were performed using the same condition in the presence of 2 μM or 4 µM of protein when alone, and 2 μM of protein A +2 µM of protein B when protein were mixed.

Native gel mobility shift assays - sigma 54 interaction assay

Gel mobility shift assays were conducted to detect protein-protein complexes. Assays were performed in a 10 µl final volume containing: 10 mM Tris-acetate (pH 8.0), 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM DTT, 4 mM ADP, ± NaF (5 mM) ± 32P-HMK-σ54 (1 µM). Where required, PspF1-275 WT or variant (5 µM) ± AlCl3 (0.4 mM) were added for a further 20 minutes at 37°C. Complexes were analysed on a native 4.5% polyacrylamide gel. Radiolabelled HMK-σ54 was measured by PhosphorImager (Fuji Bas-1500) and analysed using the Aida software.
**In vitro** Open Complex formation and full-length transcription assays

Open complex formation assays were performed in a 10 µl volume containing: 10 mM Tris-acetate (pH 8.0), 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM DTT, 4 mM dATP, 0.1 µM core RNAP enzyme, 0.4 µM σ54 and 20 nM promoter DNA (supercoiled *S. meliloti nifH* promoter). The mix was pre-incubated at 37°C for 5 minutes and the reaction started by addition of 5 µM of PspF1-275 WT or variants and incubated for varying times at 37°C.

Open complex formation was monitored following the synthesis of the transcript (-1 UpGGG+3) started by simultaneous addition of heparin (100 µg/ml), initiating dinucleotide UpG (0.5 mM), GTP (0.01 mM) and 4 µCi [α-32P]GTP for a further 10 minutes. The reaction was stopped by addition of loading buffer and analyzed on a 20% denaturating gel. Full-length transcription was initiated by adding a mix containing 100 µg/ml heparin, 1 mM ATP, CTP, GTP, 0.5 mM UTP and 3 µCi [α-32P]UTP for a further 10 minutes. The reaction was stopped by addition of loading buffer and analyzed on 6% sequencing gels.

Radiolabelled RNA products were measured by PhosphorImager (Fuji Bas-1500) and analysed using the Aida software.

**Gel filtration through Superdex 200**

PspF1-275WT and variants (at different concentrations) were incubated for 5 minutes at 4°C in buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl and 15 mM MgCl2, ± 0.5 mM ATP or ADP where indicated. 50 µl samples were then injected onto a Superdex 200 column (10 × 300 mm, 24 ml, GE Healthcare) and equilibrated with the sample buffer ± nucleotide. Chromatography was performed at 4°C at a flow rate of 0.5 ml/min and columns were calibrated with globular proteins: thyroglobulin (669 kDa), apoferritin (443 kDa), b-amylase (200 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). All experiments were repeated at least twice and the elution profiles obtained were similar. Proteins were detected at 280 nm.

**β-galacosidase assays**

To perform *in vivo*, β-galacosidase assays, variant constructs were sub-cloned from pET28b to pBAD18C using XbaI/HindIII restriction enzyme. After transformation into cells containing a *pspA-lacZ* reporter construct inserted on the chromosome, cells were grown overnight at 37°C in LB broth containing the appropriate antibiotic and then diluted 100-fold (initial OD600≈0.025) into the same medium (5 ml). Following incubation to OD600≈0.30, cultures were induced with arabinose for 1 h, further grown to mid-exponential phase (OD600≈0.5-0.6) and then assayed for β-galactosidase activity as described by (Miller, 1972). Enzyme activities (in Miller units) represent the means ± SD of the triplicate average values from at least six independent cultures.
Table S1, related to Table 1: Plasmids used in this work.

|   | in pET28b | reference |
|---|-----------|-----------|
| WT | pPB1 | (Bordes et al., 2003) |
| L9A | pNJ38 | this study |
| K42A | pNJ39 | (Schumacher et al., 2004) |
| E43A | pNJ40 | this study |
| E43D | pNJ41 | this study |
| E43Y | pNJ42 | this study |
| L44A | pNJ43 | this study |
| N64Q | pNJ44 | (Joly et al., 2008) |
| D107A | pNJ45 | (Schumacher et al., 2004) |
| E108A | pNJ46 | (Joly et al., 2007) |
| E108Q | pNJ47 | (Joly et al., 2007) |
| E118A | pNJ48 | this study |
| E118D | pNJ49 | this study |
| E118R | pNJ50 | this study |
| R122A | pNJ51 | this study |
| R122E | pNJ52 | this study |
| E125A | pNZ1 | this study |
| E125D | pNZ2 | this study |
| E125Q | pNZ3 | this study |
| Y126A | pNJ54 | this study |
| Y126E | pNJ55 | this study |
| R162A | pNJ56 | (Schumacher et al., 2004) |
| R162E | pNJ57 | this study |
| R162H | pNJ58 | this study |
| R162K | pNJ59 | this study |
| D164A | pNZ4 | this study |
| D164N | pNZ5 | this study |
| D164Q | pNZ6 | this study |
| R168A | pNJ60 | (Schumacher et al., 2004) |
| R168E | pNJ61 | this study |
| R168H | pNJ62 | this study |
| R168K | pNJ63 | this study |
Figure S1, related to Figure 1: Oligomerization of PspF variants. Gel filtration on Superdex 200 was performed at 4°C. The scale bars give the scale of ordinate axis; absorption units (AU) correspond to an $A_{280\text{nm}}$ of 1.

Oligomerization is one of the conserved properties of AAA+ proteins needed for the formation of the active catalytic site at the interface between subunits. We observed that the substitution of almost all the residues targeted in this study (except for L9 and E125) have a major effect on the oligomerization, favouring formation of a hexameric state similar to the one observed for the WT in the presence of nucleotide. This outcome is in complete agreement with studies suggesting that the PspF hexamer exists in a conformation non-functional for stable binding $\sigma^{54}$ in the absence of nucleotide, insured by the complex interaction network between the residues located at the level of the interface (Joly and Buck, 2010). Substitution of the residues taking part in this network will “unlock” the conformation and the hexamer will adopt an “activate conformation”. In the case of L9A and E125D, the oligomer formation is reduced compare to WT, but is still dependent on the protein concentration and/or on the presence of nucleotide.
A. Figure S2, related to Figure 3: ATPase activity of the mixed PspF between \textit{A. \textit{cis}} (N64, D107 and E108) and all variants used in this study, \textit{B. \textit{cis}} (N64, D107, E108 and Y126) and E43 variants. Histograms represent the \( k_{\text{cat}} \) observed for the different variants mixed at equi-molar concentration. Experiment was performed three times independently and the maximal error observed was below 10%.

\textit{C. preliminary experiment showing in vivo activity of the PspF variants}. We used \( \beta \)-galatosidase activity assay of PspF\(1 - 275\) variants E125, R162, D164 and R168 overproduced in MG1655 \( \Delta \text{pspA}\Delta \text{pspF} \) strain with chromosomal fusion of \textit{pspAp-lacZ}.

We tested the effect of the substitution of few residues of PspF on its activity \textit{in vivo} using a reporter strain harboring a \textit{pspAp-lacZ} fusion on the chromosome and overproduced PspF variants. As shown in the preliminary results presented in the Figure S2SC, the activities of the variants tested are (when detected) very low and are consistent with the \textit{in vitro} data. It is not possible to readily distinguish from the \textit{in vivo} experiment whether the observed promoter activity reflects a reduction of the specific PspF activity or a reduction in the amount of active protein. As observed
during protein purification, the substitution of a residue located at the interface between subunit often affects the solubility of the protein. Another limitation preventing us from conducting any *in vivo* "mixing" experiment, in the presence of two complementary substitutions, is the probable relative differences in the production of the variants in the same cell at the same time. Hence we did not perform a thorough systematic *in vivo* analysis with all the variants. Rather, *in vitro* methods based on using defined purified soluble proteins measured the effects of these substitutions upon activities.
Figure S3, related to Figure 3: Overlayed PspF and NtrC1 structures. A- Overlayed PspF hexamer and NtrC1 heptamer models. B- Overlayed crystal structures of PspF monomer (pdb: 2C9C) and NtrC1 E239A (pdb: 3M0E) with the two arginine residues proposed to be R-fingers residues PspF R162/PspF R168 and NtrC1 R293/NtrC1 R299. Figure was generated using PyMol software.
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