Production and characterization of a recombinant single-chain antibody (scFv) for tracing the σ^{54} factor of *Pseudomonas putida*

by

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ABSTRACT

The number of alternative sigma factor molecules per bacterial cell determines either stochasticity or evenness of transcription of cognate promoters. An approach for examining the abundance of sigmas in any sample of bacterial origin is explained here which relies on the production of a recombinant highly specific, high-affinity single-chain variable Fv domain (scFv) targeted towards unique protein sites of the factor. Purposely, a super-binder scFv recognizing a distinct epitope of the less abundant sigma σ^{54} of *Pseudomonas putida* (also known as σ^{N}) was obtained and its properties examined in detail. To this end, an scFv library was generated from mRNA extracted from lymphocytes of mice immunized with the purified σ^{54} protein of this bacterium. The library was displayed on a phage system and subjected to various rounds of panning with purified σ^{54} for capturing extreme binders. The resulting high-affinity anti-σ^{54} phage antibody (Phab) clone named C2 strongly attached a small region located between positions 172 and183 of the primary amino acid sequence of σ^{54} that overlaps its core RNA polymerase-binding region. The purified scFv-C2 detected minute amounts of σ^{54} in whole cell protein extracts not only of *P. putida* but also *Escherichia coli* cells and putatively in other bacteria as well. The affinity constant of the purified antibody was measured by surface plasmon resonance (SPR) and found to have a K_D (k_{off}/k_{on}) in the range of $2x10^{-9}$ M. The considerable affinity and specificity of this recombinant antibody makes it a tool of choice for quantitative studies on gene expression of σ^{54}-dependent promoters in *P. putida* and other Gram-negative bacteria.

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1. Introduction

The current onset of systems biology, single-cell methods and synthetic approaches has brought new perspectives to long-time recurrent questions on how the flow of gene expression from DNA to biological activity proceeds in both bacterial populations and individual cells (Swain et al., 2002). One of the most intriguing issues raised by contemporary research is that of non-homogenous transcription of given genes because of the stochastic regime that operates in the regulatory networks of single bacteria (Maier et al., 2011). Stochastic phenomena in prokaryotic transcription is predicted to exacerbate when one limiting factor happens to be in short supply and its counts vary from cell to cell (Silva-Rocha and de Lorenzo, 2010). Because of being at the summit of any prokaryotic gene expression program, the abundance or dearth of specific sigma factors is propagated through the entire regulatory network of bacteria in a fashion prone to create stochastic behaviour (Raffaelle et al., 2005; Ghosh et al., 2010; Silva-Rocha and de Lorenzo, 2010). There is a variety of such sigma factors that occur forming complexes with the bacterial core RNA polymerase (RNAP) and which are responsible for promoter recognition specificity (Ishihama, 2000). Most of these σ factors belong to a single class that show a considerable similarity to the major, housekeeping σ factor of *E. coli* (σ^{70}; Wosten, 1998). Cognate promoters include those for expressing general maintenance functions as well as others for dealing with special conditions e.g. heat-shock stress, sporulation. Sigmas of the σ^{70}-related class appear to be abundant and thus less prone to cause variations *per se* in gene expression among cells in a population (although other factors may become limiting in given promoters). In contrast, many eubacteria have also a separate class of σ factors that is composed by a unique member (σ^{54}, encoded by *rpoN*) that differs both in amino acid sequence and mechanism of transcription (Buck et al., 2000). Such σ^{54} is a key element in the activation of promoters implicated in a diversity of specialized biological processes in many Gram-negative bacteria: energy metabolism, utilization of alternative nitrogen and carbon sources, expression of flagella and chemotaxis, production of secretins and several others (Reitzer and Schneider, 2001). In the genus *Pseudomonas*, the *rpoN* gene occurs in a single copy per genome and its expression seems to be constitutive and subject to negative auto-regulation (Kohler et al., 1994). Many strains of *P. putida* and other soil bacteria have a versatile metabolism for utilization of alternative carbon source, including aromatic compounds such as xylenes or phenols (Shingler, 2011). These biodegradative genes are often under the control of σ^{54}-dependent promoters (Carbajosa et al., 2009).

During the course of our system-level analysis of the expression of the transcriptional network that determines expression of *m*-xylene biodegradation in *P. putida* mt-2 (Koutinas et al., 2010; Silva-Rocha et al., 2011) we faced the necessity of measuring the absolute number of σ^{54} molecules per cell. This is because the two substrate-responsive master promoters of the system, named *Pu* and *Ps* are transcribed by the σ^{54}-containing form of the RNAP in concert with a matching enhancer-binding protein called XylR (Jurado et al., 2003). The approach for tackling this issue disclosed below relies on the production of a specific recombinant antibody with an affinity for σ^{54} which is high enough for detecting very low levels of the protein. Although the same could be in principle be brought about by a standard monoclonal derived from a hybridome (Anthony et al., 2003) we wished to exacerbate the affinity-to-be. This is feasible if one produces a large library of the hypervariable antigen-binding domain of immunoglobulins (Ig) in the format of a single-chain (scFv) molecular species and then selects for super-binders. scFvs are composed of a single polypeptide that embodies the whole binding surface which is shaped naturally by two polypeptides at the tips of the two arms of the Ig molecule. Production of scFvs requires the gene segments encoding the variable domains from the heavy (V_H) and light (V_L) chains of Igs (Plückthun, 1996; Winter et al., 1994) to be amplified separately and then assembled *in vitro* as a single polypeptide chain (Fig. 1). This is made by means of a linker encoding a flexible peptide that allows cloning of the whole DNA sequence in a phagemid vector for *in vivo* packaging in M13 capsides and subsequent display of the scFv fused to the phage coat protein III. The angle here is that the physical association in a phage particle of the scFv antibody with its encoding gene allows the iterative enrichment of best binders (*phage display*; Hoogenboom, 1997). As shown below, this technique has been instrumental for isolating an scFv clone with an affinity for the σ^{54} factor of *P. putida* that is high enough for detecting the very limited pool of molecules of the factor that occurs in cells of this species and other bacteria (Jishage et al., 1996).

2. Materials and Methods

2.1. Bacteria, phages, growth and induction conditions

The *E. coli* strain XL-1 Blue (*recA1 gyrA96 relA1 endA*1 *hsdR17 supE44 thi1 lac*; F'*proAB lacI*^{q} *lac*ZΔM15 *Tn10*; Tc^R; Stratagene) was used as the host for bacteriophages and phagemids. Alternatively,

E. coli XL-1 Blue cells were routinely grown at 30º C in 2xYT liquid media, or LB-agar plates, containing glucose (2% v/v) for repressing the *lac* promoter, 10 μg/ml of tetracycline (Tc) for F' selection, and 150 mg/ml of ampicillin (Ap) for phagemid selection. For packaging of phagemids into M13 capsides, these *E. coli* cells were infected with VCS-M13 helper phage (Km^R; Stratagene). Amplification of this VCS-M13 helper was carried out in *E. coli* XL-1 Blue cells grown at 30 ºC in 2xYT media containing 50 μg/ml of kanamycin (Km). The non-supressor strain *E. coli* HB2151 was employed for the purification of scFv C2 from the periplasm. The strain harbouring the phagemid pPC2 encoding the scFv named C2 (see below) was grown in LB with ampicillin (Ap, 150 μg/ml) at 30 ºC. Glucose (2% v/v) or IPTG 0.5 mM were used for repression or activation respectively of scFv C2 expression. *E. coli* strain BL21/DE3 (*ompT hsdS*$_B$ r_B^- m_B^- *gal dcm* lDE3; Novagen) transformed with plasmid pLysS was employed for the production of σ^{54} fragments encoded by DNA sequences cloned in pET-derivatives (Novagen). These were propagated in *E. coli* DH5αF' [Δ(*lacZYA-argF*) U169 ϕ80 (*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 relA1 supE44 thi,* F']. *E. coli* BL21/DE3 and DH5αF' strains were grown at 37 ºC in LB media (Miller, 1992) containing appropriate antibiotics. Chloramphenicol (Cm; 30 μg/ml) and Ap (150 μg/ml) were employed for selection of pLysS and pETs, respectively. The production of σ^{54} fragments in *E. coli* BL21/DE3 (pLysS) cells, harboring given pET-derivatives was induced by addition of 1.0 mM isopropyl-1-thio-β-D-galactoside (IPTG) to mid-log phase (OD_{600} ~0.5) cultures. After 2 h induction, *E. coli* cells were harvested from the cultures and analysed by SDS-PAGE and Western blot (see below). *Pseudomonas putida* strain KT2442 was grown at 30 ºC in LB medium. *E. coli* TH1 *(ΔlacU169, ΔrpoN*) (Ow and Ausubel, 1983) and its isogenic wild type strain (*E. coli* YMC9) were used as controls.

2.2. Phagemids, plasmids and DNA constructs

Standard methods were used to purify, analyse, manipulate, and amplify DNA (Ausubel, 1994). All oligonucleotides were synthesized by Isogen Bioscience BV. DNA constructs and phagemids were sequenced using the Dideoxy method (ABI-PRISM automated DNA sequencer, Perkin Elmer). The phagemid pCANTAB-5Ehis (Ap^R; Fernandez et al., 2000) was utilized for cloning and expression of the DNA sequences encoding scFv domains. The control phagemid pHen-MBP (Ap^R; Nissim et al., 1994) determines an scFv against the *E. coli* maltose binding protein (MBP). To construct the pET-vectors expressing truncated portions of σ^{54}, the DNA fragments encoding the corresponding deletions were

amplified by PCR from plasmid pNTR1, which carries the wild-type *rpoN* gene *from P. putida* in a 3.4 kb *Sal*I fragment cloned into pUC8 (Köhler, 1990). The primer 5'-GGAAGAAGGAGATATA CCATGGCTAAACCATCGCTCGTCCTAAA-3' was employed for amplification of PCR fragments 1, 3, 4, and 5 (see below) in combination with one of the following primers: 5'-ATGATGATGTGCGGCCG CGGCGTATTGTGGGTTGA-3' for fragment 1; 5'-ATGATGATGTGCGGCCGCGCGCTTGGC TTCGGTCA-3' for fragment 3; 5'-ATGATGATGTGCGGCCGC GCAGCAACAGGCATTCG-3' for fragment 4; and 5'-ATGATGATGTGCGGCCGCGGACATCGGTGCCAGGT-3' for fragment 5. For amplifying fragment 2, the primers 5'-GGAAGAAGGAGATATACCATG GAAGCCATCCCACGCCT-3' and 5'-ATGATGATGTGCGGCCGCCATCAGTCGCTTGCGTT-3' were employed. The resulting DNA fragments were used as templates for a second amplification with primers T7RBS1 (5'-CCGAATTC TAATACGACTCACTATAGGGAAGAAGGAGATATA-3') and HISTAIL (5'-CGCGGATCCTCAGTGATG GTGATGATGATGTGCGGC-3') that introduced *Bam*HI and *Not*I restriction sites and a polyhistidine tag (6xhis) at the extremes of the primed segments. The PCR products obtained by amplification of fragments 1 and 2 were then cloned at the *Nco*I and *BamH*III sites of pET11d (Novagen) for expression of truncated protein F1 and F2. Similarly, the DNA bearing the thereby amplified sequences 3, 4, and 5 were cloned into the *Nco*I and *Not*I pET21d (Novagen) for expression of protein fragments F3, F4, and F5.

2.3. Immunizations

Three female Balb/c mice were immunized by intraperitoneal (i.p.) injection with σ^{54} of *P. putida* cells purified by Sepharose chromatography (Cannon et al., 1996; the kind gift of F. Bartels). To this end, the σ^{54} protein was dialysed against phosphate buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 137 mM NaCl, pH 7.0) and diluted to 0.5 mg/ml in PBS containing SDS 0.1% (w/v). Just prior to injection, 0.3 ml of this protein stock were mixed with an identical volume of Ribi adjuvant (MPL+TDM™, Sigma) previously reconstituted in PBS at 1 mg/ml. 0.2 ml of this antigen/adjuvant emulsion (corresponding to 40 mg of σ^{54}) was then injected i.p. (Harlow and Lane, 1988) to each mouse at days 0, 21 and 42. Ten days after the last injection, a ~100 µl blood sample was taken from each mouse, and the sera tested in ELISA (Harlow and Lane, 1988) for determining the specifc Ig response elicited against σ^{54}.

2.4. Production of a scFv phage display library

The protocols described by McCafferty and Johnson (1996) were followed for generation and testing of large scFv pools with the modifications specified in Fraile et al. (2001). Briefly, the spleens of inoculated mice were processed for extraction of mRNA following the guanidinium isothiocyanate-acid phenol procedure (Ultraspec™ RNA isolation, Biotecx). The poly-A^+ mRNA was purified using an oligo-T resin (OligoTex™ kit, Qiagen) and then employed as template for a first-strand cDNA synthesis reaction (GE Amersham). The V_H and V_L gene segments were amplified from the cDNA samples using the primers indicated in (Fraile et al., 2001). The scFv-genes were then joined in a V_H-linker- V_H configuration. After amplification, the ~0.7 kb DNA fragments corresponding to the assembled scFvs were digested with *Sfi*I and *Not*I restriction enzymes, gel-purified, and ligated into the same sites of pCANTAB-5Ehis (Fernandez et al., 2000) in such a way that the scFvs sequences become tagged with a an E-tag epitope and expressed as a fusion to the phage M13 gp3 coat (pIII) protein. The recombinant library was electroporated into *E. coli* XL1 Blue cells and $2x10^6$ independent colonies were harvested from these plates in LB + glycerol (15% v/v), pooled, and stored at -80 ^oC. A control re-ligation of the *Sfi*I/*Not*I pCANTAB-5Ehis vector used gave ~1% transformants.

2.5. Rescue and selection of phagemids

Assembling the library of M13 particles displaying scFv-protein III hybrids (Phab production) was done as described in (Fraile et al., 2001) and sketched in Fig. 1. For large-scale preparation of monoclonal Phab(s), a single colony of *E. coli* XL-1 cells harbouring the phagemid clone under examination was inoculated and the same protocol was followed. The production scFv-protein III fusions during viral packaging was tested by immunoblotting of *E. coli* whole-cell protein extracts (see below). For rapid screening of σ^{54} Phab binders, a small-scale rescue of phagemids was performed in cultures of *E. coli* cells grown in 96-well microtitre plates and selection of those binding σ^{54} made with the panning procedure described in (Fraile et al., 2001). To this end, purified σ^{54} (2.5 mg/ml) was adsorbed to microtiter immunoplate (Maxisorb™, Nunc). The bound Phabs were later used to infect *E. coli* XL-1 Blue cells and plated on 2xYT media with glucose, Ap and Tc. After 24 h incubation at 30 ^oC, the resulting colonies were harvested as a pool and used for phagemid rescue. The thereby generated Phab sub-

library was then subject to two additional rounds of panning for enrichment of strong σ^{54}-binders. Finally, individual Phab clones were rescued at a small scale and their specific binding to σ^{54} determined by ELISA.

2.6. Purification of the scFv C2

Plasmid pPC2 was transformed into the non-suppressor *E. coli* strain HB2151. 250 ml of LB, containing the appropriate antibiotics were inoculated with this strain and grown at 25 ^{o}C. When the culture reached the OD_{600} ~1.0, IPTG was added to a final concentration of 0.5 mM, and the culture was further incubated for 16 h. Periplasmic protein extracts were prepared following the osmotic-shock method (Harrison et al., 1996) with minor modifications. For this, the *E. coli* cells from the overnight-induced culture were spun down and resuspended in 20 ml of a shock-buffer containing 30 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20% w/v sucrose, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and a cocktail of protease inhibitors (Complete™, Roche). After 20 min incubation at 4 ^{o}C, the sample was centrifuged (8000g, 15 min, 4 ^{o}C) and the supernatant (S1) separated from the sedimented biomass. This was resuspended in 20 ml of $MgSO_4$ 5 mM, and centrifuged again as above. This second supernatant (S2) was combined with S1 to obtain the periplasmic proteins extract. This fraction was dialysed against buffer DI (40 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1mM PMSF). The sample was then centrifuged (6500 g, 10 min, 4 ^{o}C) to discard any particulated material and applied onto a chromatography column loaded with a Cobalt-containing agarose resin (TALON™, Clontech). The matrix was washed once, first with 40 ml DI buffer and then with 20 ml DI buffer containing 5 mM imidazole pH 8.0. The bound His-tagged scFv C2 was subsequently eluted from the column by adding 15 ml of buffer DI with 100 mM imidazole at pH 8.0. Those 1 ml fractions containing the scFvs (as determined by immunoblots using anti-E-tag-MAb-POD) were pooled and dialysed against buffer DII (40 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1 mM EDTA, 5 % glycerol). The concentration and purity of scFv C2 was determined by silver staining of SDS-polyacrylamide gels using as standards serial 4-fold dilutions of broad range molecular weight markers (Bio-Rad).

2.7. Enzyme-linked immunosorbent assays (ELISA)

ELISAs were performed at room temperature in 96-well immunoplates (Maxisorb™, Nunc) adsorbed for 2 h with either σ^{54} or ovalbumin (Sigma) suspended in 50 mM $NaHCO_3$ pH 9.0 at a concentration of 2.5 mg/ml. Excess of antigen was washed out and the plates blocked for 2 h using 200 µl per well of MBT-buffer. The blocking solution was discarded and the primary antibodies (immune sera, Phabs, or purified scFvs at different concentrations) were added to the wells (50 µl of the indicated dilution in MBT-buffer). After 1 h of incubation, the unbound Igs, Phabs, or scFvs were removed by four 3-min washings of the wells with PBS, 0.1% v/v Tween 20. Detection of the bound antibodies followed a different procedure in each case: goat anti-mouse IgG-POD conjugate (Sigma) for Igs, anti-M13-MAb-POD conjugate (GE Amersham) for Phabs and anti-E-tag MAb-POD conjugate (GE Amersham) for scFvs. After 1 h incubation with these secondary antibodies (all of them at 1:5000 dilution in MBT buffer: 3% skimmed milk, 1% bovine serum albumin, and 0.1% Tween 20 in PBS), microtiter plates were washed as before and developed using a mixture (80 µl per well) of *o*-phenylenediamine (0.4 mg/ml; OPD, Sigma) and H_2O_2 (0.012% v/v; Sigma) in phosphate-citrate buffer pH 5.0 (103 mM dibasic sodium phosphate, 24 mM citric acid). The reaction was then allowed to proceed in the dark for 10 min, stopped with 0.6 N HCl (20 µl of 3 N HCl per well), and the OD_{490nm} of the plates determined (Benchmark™ microplate reader, Bio-Rad). Background binding to ovoalbumin (usually $OD_{490nm} \leq 0.05$) was subtracted from the values of specific antigen-binding obtained in all cases.

2.8. Protein analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard protocols using the Miniprotean™ system (Bio-Rad). Whole-cell protein extracts were prepared by harvesting the cells (10000 g, 5 min) from 1-20 ml of cultures (depending of the OD_{600}) in LB and resuspending the pellets in 100 µl Tris HCl 10 mM pH 7.5. Next 2X SDS-sample buffer (Tris-HCl 120 mM pH 6.8, SDS 2% w/v, glycerol 10% v/v, bromophenol blue 0.01% w/v, 2-mercapto-ethanol 2% v/v) was added to the samples, boiled for 10 minutes, sonicated briefly (~5 sec) and centrifuged (14000 g, 10 min) to eliminate DNA viscosity, cell debris and other particulated material. Samples with thereby prepared extracts equivalent to ~10^8 cells were loaded per lane. Prestained standards (Kaleidoscope™, Bio-Rad) were used as markers of known molecular weight for the SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) using a semi-dry

transfer apparatus (Bio-Rad). Following protein transfer, the membranes were blocked for 2 h at RT (or for 16 h at 4º C) using MBT-buffer. For immunodetection of σ^{54} with the purified recombinant antibody, membranes were incubated with 20 ml of MBT-buffer containing 500 ng of scFv C2. Unbound antibodies were eliminated by four washing steps of 5 min in 40 ml of PBS, 0.1% (v/v) Tween 20. Next, anti-E-tag-MAb-POD conjugate (1:5000 in MBT-buffer, 1 mg/ml in MBT-buffer; Amersham Pharmacia Biotech) was added for detecting the bound scFvs. After 1 h incubation, the membranes were washed four times with PBS/0.1% (v/v) Tween 20, and the bound peroxidase-conjugates developed by a chemiluminiscence mixture of 1.25 mM luminol (Sigma), 42 mM luciferin (Roche) and 0.0075% (v/v) H_2O_2 in 100 mM Tris HCl pH 8.0. BM Chemiluminiscence Blotting Substrate POD (Roche) was subsequently used for developing the peroxidase-conjugates. The membranes were soaked in this mixture for 1 min in the dark and immediately exposed to an X-ray film (X-OMAT™, Kodak). For fine-mapping of the epitope targeted by scFv C2, a collection of deca-peptides and dodeca-peptides covering residues 168 to 183 of the σ^{54} sequence and bearing the alanine substitutions indicated in each case were synthesized by spot synthesis (Abimed, Langerfeld, Germany) onto amino-derivatized cellulose membranes (Frank and Overwin, 1996; Reusch et al., 1994). Membranes were then incubated with scFv C2 and developed with anti-E-tag-MAb-POD as explained in (Fraile et al., 2001).

2.9. Affinity measurement by Biacore

The affinity and kinetic parameters of binding were determined using surface plasmon resonance in a BIAcore 1000 instrument (GE Healthcare). The purified σ^{54} antigen was covalently coupled to the carboxymethylated dextran polymer attached to a gold surface of the BIAcore sensor chip CM5 at 1547 resonance units. Different concentrations (25, 50, 100, and 200 nM) of purified scFv C2 were then injected at a flow rate of 20 ml/min in buffer 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, and 0.1 mM EDTA until reaching steady state (k_{on}). The same buffer was injected at identical flow rate for washing bound scFvC2 (for k_{off} determination). Binding specificity was confirmed by the injection of an unrelated scFv. The sensor chip was regenerated between the difference cycles with 20 mM glycine-HCl pH 2.5. Kinetic binding constants k_{on}, k_{off}, K_D (k_{off}/k_{on}) were determined using Biacore BIA-evaluation software from the different sensograms obtained with each concentration of the analyte.

2.10. Molecular modelling

The 3D simulation of the scFv C2 structure was built with the homology-modelling software SWISS-MODEL available at http://swissmodel.expasy.org/ (Arnold et al., 2006). The segment A135 to N277 of the *P. putida* σ^{54} protein containing the cognate target sequence was modelled using as a reference the physical 3D structure of an overlapping homologous fragment of the same sigma factor of *Aquifex aeolicus* (Hong et al., 2009). The plausible interaction between the two proteins was visualized with the software HADDOCK http://haddock.science.uu.nl/ (de Vries et al., 2010) in which the contact surfaces were restricted to the 6 CDRs (complementarity determining regions) of scFv C2 and the segment YLEDTLEEISAG of σ^{54}.

3. Results and Discussion

3.1. Selection of a phage antibody against σ^{54} of P. putida

A library of ~$2x10^6$ independent scFv clones was obtained from mice immunized with purified σ^{54} of *Pseudomonas putida* and displayed on the capsid of M13 phages as explained in the Materials and Methods section above. These phages were employed in a panning procedure to select clones binding purified σ^{54}. Three rounds of selection and amplification of the bound phages were performed. In each round, the input phage titers were kept uniformly at $2x10^{11}$ PFU and after each selection the number of phages bound to σ^{54} was determined. The titer of phages bound to σ^{54} steadily increased from ~10^4 plaque forming units (PFUs) in the first and second round to ~3 $x10^8$ PFU in the third round, indicating a selective amplification of certain phage-antibody specimens (Fig. 2A). 96 clones of the thereby amplified scFv-phage particles (Phabs) were individually rescued and their binding properties tested by ELISA. Out of this screening, 30 individual phagemid clones were found to manifest a strong and specific reactivity against purified σ^{54}. Their DNA sequence revealed that 25 of them encoded the very same scFv (hereafter referred as scFv C2, born by phagemid pPC2). Two other different scFvs clones were found among the amplified Phabs (referred as C23 and C47), but showed lower affinity for σ^{54} in ELISA when tested individually (Fig. 2B) and were not further characterized. The amino acid sequence of scFv C2 shown in Fig. 3 indicates the complementarity determining regions (CDR) of the V_H and V_L domains as identified with the Kabat numbering scheme (Johnson and Wu, 2001).

Note that the selection of scFv-phage particles is made under conditions where only strong binders can endure the various washings involved in the panning procedure. This is because the scFv attached to the apical pIII protein of the viral capsid represents just a minute part of the large M13 particle that is ultimately retained by the antigen (σ^{54}) that coats the ELISA plate. The issues at stake are therefore [i] whether the binding of the Phabs can be traced entirely to the scFv moiety of the whole macromolecular viral complex, if so [ii] whether the binding of the isolated scFv excised from the Phab retains a high affinity for the target, [iii] what are the parameters of the interaction and [iv] how specific the scFv-σ^{54} recognition is. These outstanding questions are addressed in the sections below.

3.2. Binding affinity of scFv C2 for σ^{54}

To study the strength of the interplay of scFv C2 with σ^{54}, the phagemid clone pPC2 encoding the antibody under examination was entered in a specialized *E. coli* strain which expressed the linked V_H-V_L fusion of the parental Phab as a single E-tagged and His-tagged polypeptide secreted into the periplasm of the host cells. This was brought about by the characteristics of the cloning vector employed for rescuing the V_H-V_L sequence library (pCANTAB-5Ehis; Fernandez et al., 2000) and the use of a non-suppressor *E. coli* strain as expression host in such a way that the scFv module is produced as non-fused, stand-alone polypeptide. On this basis, scFv C2 was extracted from the periplasmic protein fluid of *E. coli* cells transformed with pPC2 by immobilized metal-affinity chromatography as described in the Materials and Methods section. The binding properties of the purified antibody were tested in ELISA assays, where it manifested a very strong binding to purified σ^{54} (Fig. 4A). In contrast, no significant binding to σ^{54} was observed with a control scFv (6AC3) purified from *E. coli* cells by the same procedure (Fernandez et al., 2000). No detectable binding was detected either when the plates were coated with an unrelated protein e.g. ovoalbumin.

To quantify the actual affinity of scFv C2 for the purified σ^{54} of *P. putida* we resorted to an extraordinarily sensitive surface plasmon resonance (SPR) assay run in a BIAcore platform. To this end, the native, purified σ^{54} factor was immobilized onto a gold surface covalently attached to carboxymethylated dextran. Preparations with various concentrations of purified scFv C2 were then injected and –after reaching an

steady state, washed with buffer only. The average data calculated from the corresponding sensograms (i.e. mean ± standard error) produced a k_{on} (1/Ms) *of* 3.64 ±0,35 x10^5 and a k_{off} (1/s) of 8.04 ± 0.62 x10^{-4}, what results in an apparent dissociation constant K_D (M) k_{off}/k_{on} = 2.29 ± 0.32 x10^{-9} M. Although the choice of concentrations employed in the assay was limited, these figures suggest the gross affinity of the scFv to σ^{54} to be in the low nM range. As before, no significant signals were detected when the test was run with an unrelated scFv or when the protein immobilized on the BIAcore chip was not σ^{54}. The remarkable strength of the interaction scFv C2-σ^{54} thereby visualized not only accounted for the quick enrichment of the C2 clone during the panning process but also explained the mechanical resistance of the precursor phage-antibody particle to the physical stress of the procedure.

3.2. Specificity of the scFv C2 - σ^{54} interaction

The tests above certified that the isolated scFv C2 antibody kept the high affinity for the target protein that was first detected in the corresponding Phab isolated from the panning protocol. However, they did not clarify the specificity of the interaction. This is because only two unrelated proteins were employed as controls in the ELISA and the SPR assays just discussed. Furthermore, since the plates or chips coated with the target protein probably display its amino acid sequences with various degrees of denaturation, the results of Fig. 4A do not clarify the physical form of the recognized epitope(s). To address this question, the purified scFv C2 antibody was tested in a Western blot assay in which a denaturing polyacrylamide-SDS gel was loaded with whole extracts of *P. putida* and *E. coli*. As shown in Fig. 4B, scFv C2 recognized specifically the σ^{54} protein both purified and in the context of a whole cell extract. Such recognition included the homologous protein of *E. coli*, thereby suggesting that the antibody targets distinct antigenic regions shared to an extent by the two bacteria. Finally, that binding occurred in the denaturing gel system of Fig. 4B as well as in an ELISA plate was indicative that the antibody recognized its target site in σ^{54} both as folded sequence (e. g. in the SPR assays and, to an extent in the ELISA test) and as a linear epitope. This means that at least one contiguous sequence of amino acids of σ^{54} gives a good signal with the scFv C2 antibody. To identify such a segment of the primary structure of the sigma factor we run the epitope-mapping experiments described below.

3.3. Identification of the σ^{54} site targeted by scFv C2

A low-resolution map of the part of the σ^{54} recognized by scFv C2 was drawn by examining the signals raised by a small collection of truncated polypeptides derived from the sigma factor in a Western blow assay. To this end, five DNA segments corresponding to protein fragments named F1 to F5 were amplified from the *P. putida rpoN* gene sequence and cloned into pET-vectors (Novagen) under the control of the T7 RNA polymerase promoter as explained in Materials and Methods (see Fig. 5A). Specifically, these fragments comprise amino acids Met1 to Ala324 (F1), Glu312 to Met497 (F2), Met1 to Arg238 (F3), Met1 to Leu221 (F4), and Met1 to Ser153 (F5) of the original σ^{54} primary sequence of *P. putida*. The resulting plasmids (pET-F1σ^{54} to pET-F5σ^{54}) incorporated a 6xhis-tag at the C-terminus of the respectively produced polypeptides. *E. coli* BL21/DE3 (pLysS) cells harbouring each of these pETs were induced with IPTG for overproduction of the cognate σ^{54} fragments and the extracts were loaded in SDS-polyacrylamide gel. Each of the five truncated proteins could be made out with the naked eye upon staining the polyacrylamide gel with Comassie blue and could be unequivocally identified in the blotted gel was with a anti-6xhis MAb-POD conjugate (not shown). However, those proteins labelled F2 and F5 failed to produce any signal when the same membrane was probed with purified scFv C2 in a Western assay (Fig. 5B). This was in contrast with the others (F1, F3 and F4), which were bound by the recombinant antibody as strongly as the full-length σ^{54} protein used as control. This result grossly located the target epitope somewhere between amino acids Ser153 and Leu221 of the σ^{54} primary sequence, a region that has been mapped as the RNA polymerase core binding determinant (Gallegos and Buck, 1999; Wong et al., 1994).

To delimit the amino acid sequence bound by scFv C2, overlapping deca-peptides and dodeca-peptides with either the native sequence of σ^{54} between Ser153 and Leu221 or entered with various amino acid replacements (Fig. 5C) were synthesized and attached to cellulose membranes (Frank and Overwin, 1996; Reusch et al., 1994). As shown in Fig. 5C, the peptides having the sequence YLEDTLEEIC were strongly bound by scFv C2. This epitope spans amino acid positions 172-181 of the *P. putida* σ^{54} sequence which is included in the larger region believed to interact directly with the RNA polymerase core (Gallegos and Buck, 1999; Wong et al., 1994). Furthermore, the reactivity to scFv C2 displayed by the synthetic dodeca-peptides containing amino acid replacements through the YLEDTLEEICAG segment revealed that residues Tyr172, Asp175, and (to a lesser extent) Leu177 appeared to be essential for binding. None of the other

substitutions tested had such a dramatic effect (Fig. 5C). These results identified YLEDTLEEIC as the minimal polypeptide sequence that is recognized by scFv C2 within the larger structure of σ^{54}. Furthermore, the dot-blot tests of Fig. 5C pinpointed the 3 residues mentioned above that play a key role the interactions with the CDRs of scFv C2. As explained below, these data were instrumental to make sense of the surfaces of the two proteins that are involved in mutual recognition.

3.4. A structural model for the interaction scFv C2 - σ^{54}

That scFv C2 interacts strongly with the native σ^{54} factor in the SPR assays and plausibly in the ELISA plates as well suggest that the targeted sequence had to be exposed on the protein surface. But also, since the factor is recognized as a linear, denatured polypeptide as well in a typical Western blot, chances are that the specific epitope may lack a significant secondary structure. These considerations provided a starting point for building a structural model of the entire scFv C2 - σ^{54} complex or at least of its directly interacting surfaces. Although the specific 3D structure of scFv C2 was not determined, its similarity with many other scFvs which have been crystallized makes it easy to construct a highly reliable model of the linked V_H-V_L complex that shape the recombinant antibody. On the other hand, while the physical structure of the full-length bacterial σ^{54} factor has not been resolved yet, the organization of the segment comprising residues 69-198 of the *Aquifex aeolicus* protein has been determined by NMR (Hong et al., 2009). Luckily, this protein sequence includes the positions equivalent to those found to bind σ^{54} of *P. putida* identified above, what allows a trustworthy prediction of the corresponding structure. On this basis we set out to explore an optimal fitting between the two 3D partners by applying the HADDOCK software for flexible docking simulations (de Vries et al., 2010). Fig. 6 shows the most probable spatial interaction between the inferred structures of scFv C2 and the fragment A135 to N277 of σ^{54} in which the relatively unstructured peptide YLEDTLEEICAG seems to dock exactly in the most variable region of the CDRs of the recombinant antibody. In this respect, although the data of Fig. 6 is basically a simulation, it does account for the flexibility of the scFv part to bind its target antigen either as a linear peptide or in the context of a structured protein. It has to be noted that the equivalent regions of the known σ^{54} sequences of other bacteria (*P. aeruginosa, P. syringae, B. subtillis*, *E. coli*, *S. typhimurium,* and *V. cholerae*, Fig. 7) are not fully conserved and therefore the scFv may be optimal only for the *P. putida* specimen. This does not rule out a degree of cross-reactivity with factors of related bacteria, as shown in Fig. 4B with the σ^{54} of *E. coli.*

3.5. Conclusion

The results reported above illustrate the power of phage display for isolating super-binders to distinct transcriptional factors that may be produced at very low levels in bacterial cells. The diversity of antibodies stemming from the immune response of the inoculated animal allows setting up selection (panning) conditions for scFv variants with predefined characteristics. In the case of scFv C2, the recombinant antibody seems to both interact with its target site in the *P. putida* σ^{54} protein both in the context of the native protein as well as part of a denatured polypeptide. One extra bonus of the procedure followed for isolation of scFv C2 is that one can produce large amounts of Phabs, ie. M13 capsides bearing apical scFv-pIII hybrids. These Phabs behave as antibody-like particles able to attach the entire viral body to the antigen targeted by the scFv. When Phab preparations are employed in Western blots, the proteins of interest can be revealed in the gel with an anti-M13-MAb-POD conjugate. Since the target of this reagent is the very abundant phage surface protein, there is a dramatic amplification of the corresponding signal that allows detection of just a few molecules in the sample. This approach has been employed successfully to count the number of σ^{54} molecules per cell of *P. putida* growing under various conditions, which turns out to be ~80 ± 26 (Jurado et al., 2003). This figure is in the range of that found in *E. coli* (Ishihama, 2000) and barely above the number of σ^{54}-dependent promoters (Cases et al., 2003; Zhao et al., 2010). These relatively low figures are likely to make transcription of the cellular functions controlled by the factor adopting an stochastic behaviour (Raffaelle et al., 2005; Ghosh et al., 2010; Silva-Rocha and de Lorenzo, 2010). Such a gene expression scenario in relevant promoters of *P. putida* and the TOL plasmid (Koutinas et al., 2010; Silva-Rocha et al., 2011) is currently under examination in our Laboratory.

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LEGENDS TO FIGURES

Fig. 1. Workflow for generation of scFv libraries binding the σ^{54} factor of *P. putida*. The sketch summarizes the main steps of the process as described in McCafferty and Johnson (1996). Mice were inoculated with the purified protein and, after a period of time to allow an immune reaction, RNA was directly extracted from spleens. This was first retrotranscribed to DNA and the sequences corresponding to the V_H and V_L chains amplified separately with suitable primers. These PCR products were then assembled in vitro and captured in a phage display vector. The resulting library was then subject to various rounds of panning on microtiter plates coated with the same purified σ^{54} factor used for inoculation. Those M13-scFv clones that were bound to the plates were then kept as candidates for further analysis.

Fig. 2. Phabs binding the σ^{54} factor of *P. putida.* (A) Enrichment of scFv-M13 particles upon successive panning rounds of the displayed V_H-V_H library on σ^{54}-coated microtiter plates. (B) Binding of Phabs C2, C23 and C47 and one unrelated Phab-MBP (used as a negative control; (Nissim et al., 1994)) to σ^{54} or

ovalbumin (OVA) as determined by ELISA. Different dilutions of Phabs were incubated on microtiter plates coated with the indicated antigens. After washing with PBS, the bound Phab was developed using anti-M13-POD conjugate and the plates read at OD_{490}. The data shown are relative to the maximal OD_{490} obtained by Phab C2 at the higher titer employed (OD_{490}~ 2.0).

Fig. 3. Amino acid sequence of scFv C2**.** The primary sequence of the strong σ^{54} binder named scFv C2 encoded by phagemid pPC2 is shown. The positions of the N-terminal signal peptide, the V_H domain, the $(Gly_4Ser)_3$ linker peptide, the V_L domain, and the E-tag are indicated. The complementarity determining regions (CDR) of the V_H and V_L domains are labelled and underlined. The secretion signal and the motif recognized by the bacterial signal peptidase is shown. When produced in *E. coli* XL-1 Blue cells (*supE*) this scFv is synthesized as a hybrid with the apical protein p3 of phage M13. The location of the suppressible stop codon (amber), which is placed between the scFv and the p3 coding sequence is indicated.

Fig. 4. Specific binding of scFv C2 to σ^{54}. (A) ELISA of purified scFv C2 and a negative control (scFv 6AC3; Fernandez et al., 2000) to σ^{54} and ovalbumin (OVA). Different dilutions of each of the scFvs were incubated on plates coated with the antigens indicated. After washing with PBS, the bound scFv was developed using anti-E-tag-MAb-POD conjugate and the plates were read at OD_{490}. The data shown are relative to the maximal OD_{490} obtained by scFv C2 (OD_{490}~ 2.0). (B) Western blot with purified scFv C2 made with the following samples. Lane 1 contains 1.25 ng of purified σ^{54} of *P. putida*. Lanes 2-4 are loaded with total protein extracts prepared from *P. putida* , *E. coli* wild type, and *E. coli rpoN* cells, all harvested at stationary phase. ~ 1.25 $x10^8$ CFU were applied per lane. The scFv C2 and anti-E-tag- MAb-POD were used for detection as described in the text.

Fig. 5. Mapping of the σ^{54} epitope recognized by scFv C2. (A) Five fragments derived from *P. putida* σ^{54} (F1: Met1-Ala324; F2: Glu312-Met497; F3: Met1-Arg238; F4: Met1-Leu221; F5: Met1-Ser153) were overproduced in *E. coli* BL21 (DE3) cells harbouring each of the pET-plasmids encoding them. (B) Whole-cell protein extracts were prepared from these cells and subjected to Western blot using scFv C2 as the primary antibody and the anti-E-tag-MAb-POD conjugate for developing. As shown, purified σ^{54} and fragments F1, F3 and F4 were detected, but F2 and F5 failed to give any signal. (C) Cellulose membranes

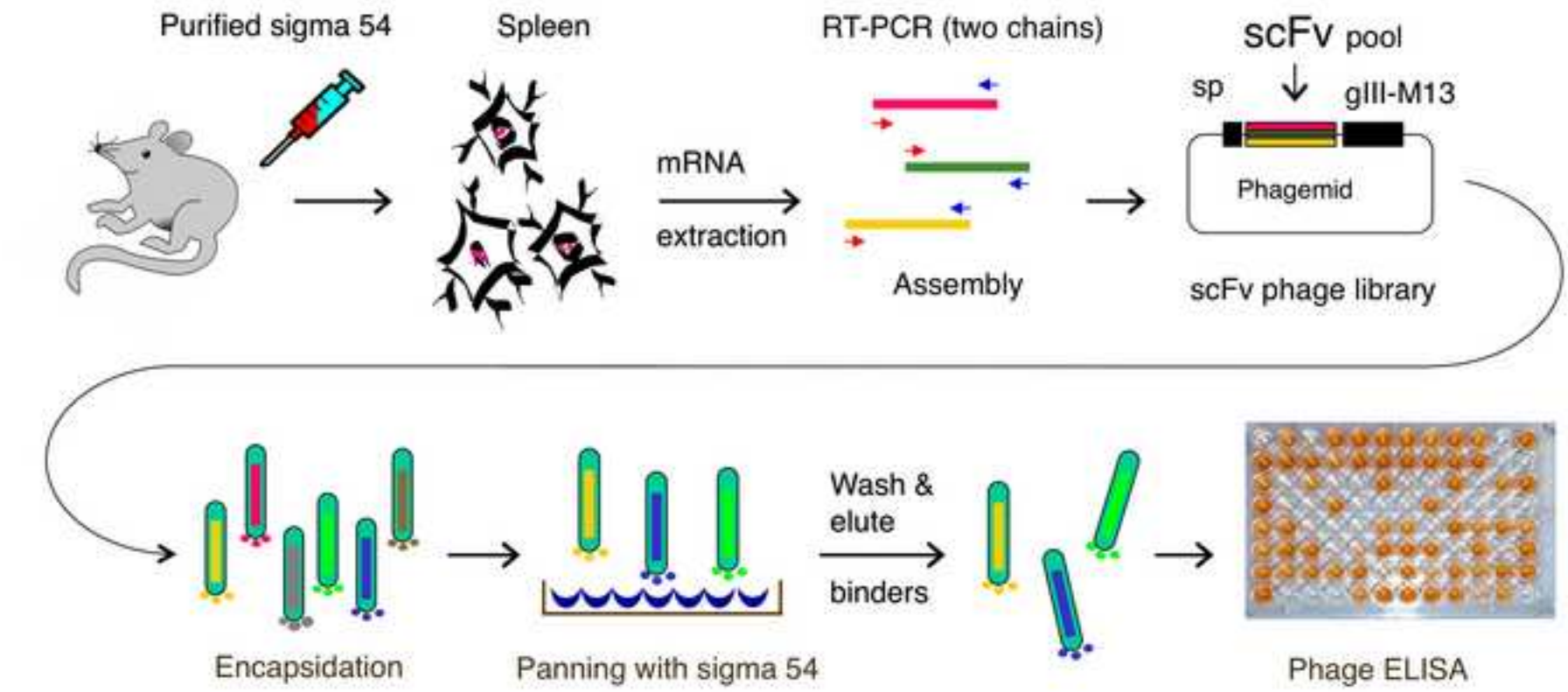
containing deca-peptides (top) spanning the σ^{54} positions Ser153 to Leu221 were probed with scFv C2 and developed with the anti-E-tag-MAb-POD conjugate. Only the peptides spanning the sequence NGQGYLEDTLEEICAG (168-184) are shown. The lower membrane was spotted with mutant dodecapeptides bearing a collection of alanine-substituted derivatives and probed with scFv C2 as before. A permissive Ser181Cys change was entered in all peptides as a reference. Note the essentiality of residues Tyr172, Asp175 and to a lesser extent, Leu177 (marked with an asterisk).

Fig. 6. Tridimensional model of the most probable interactions between the two inferred 3D structures of scFv C2 and σ^{54}. scFv C2 is coloured grey with disulphide bridges in dark blue. Fragment A135 to N277 of σ^{54} is labelled with a green code. The mapped interacting region YLEDTLEEICAG (172-183) of σ^{54} is marked in yellow. Note the apparent lack of significant secondary structure at that site.

Fig. 7. Alignment of the epitope of the *P. putida* σ^{54} protein recognized by scFv C2 with homologous regions of the factor in other bacteria. Note the conservation of the amino acids as represented with a color code. The residues more important for mutual recognition are marked with an asterisk at the bottom.

Figure(s)
Click here to download high resolution image

F1



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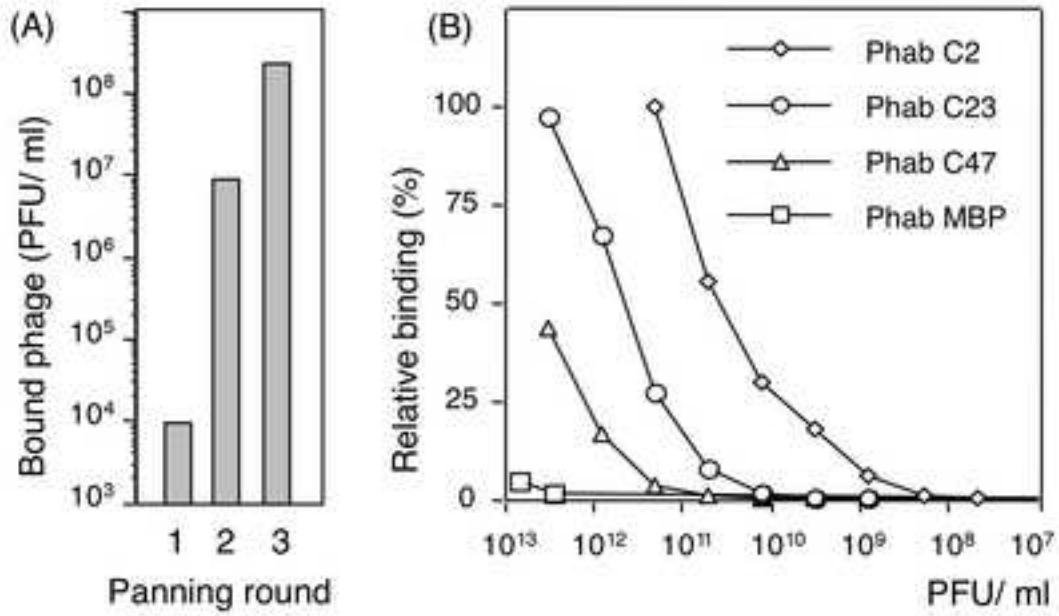

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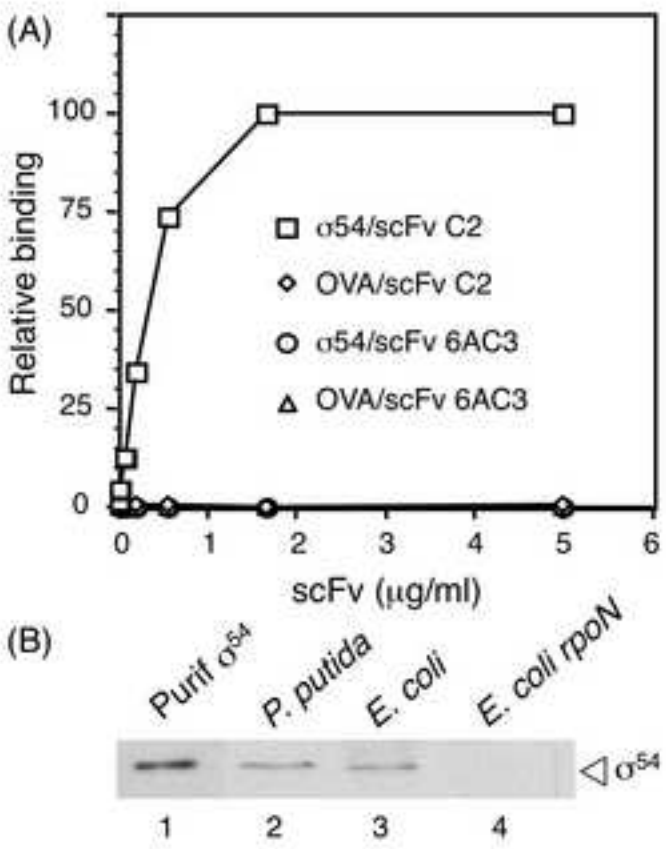

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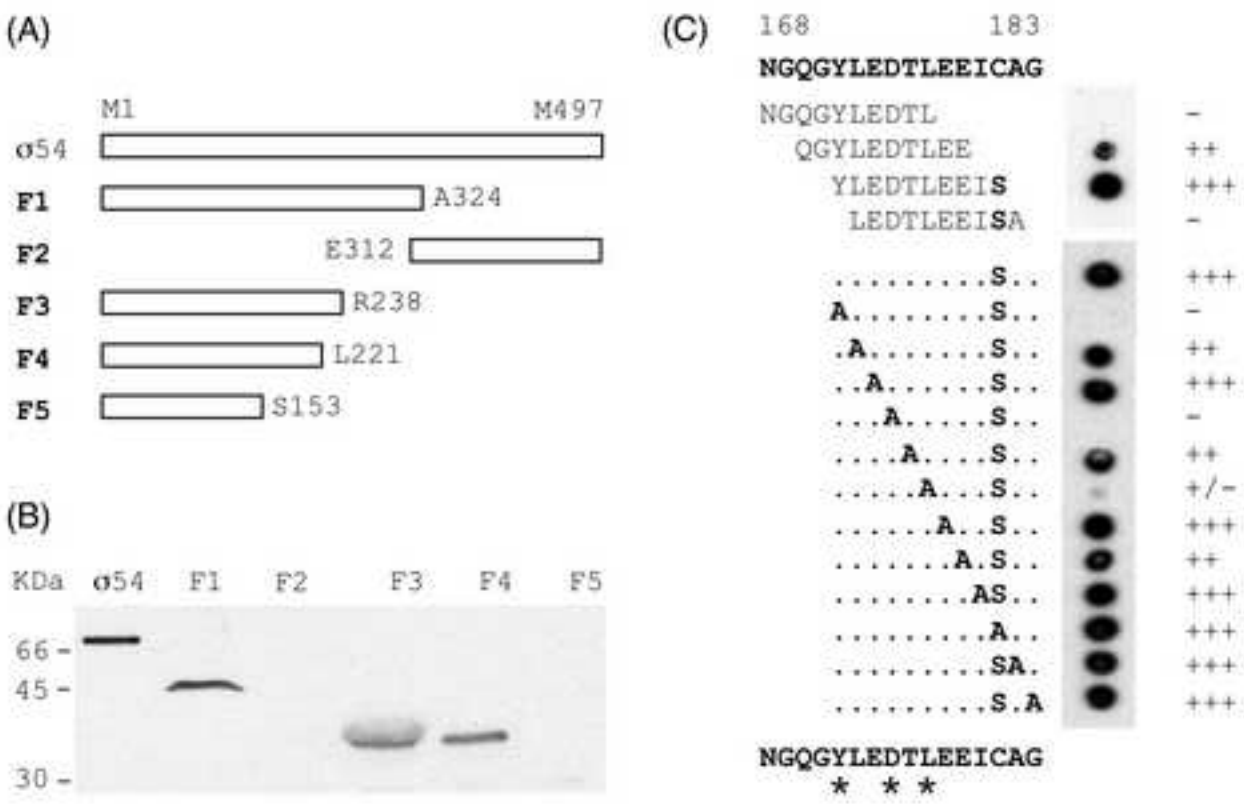

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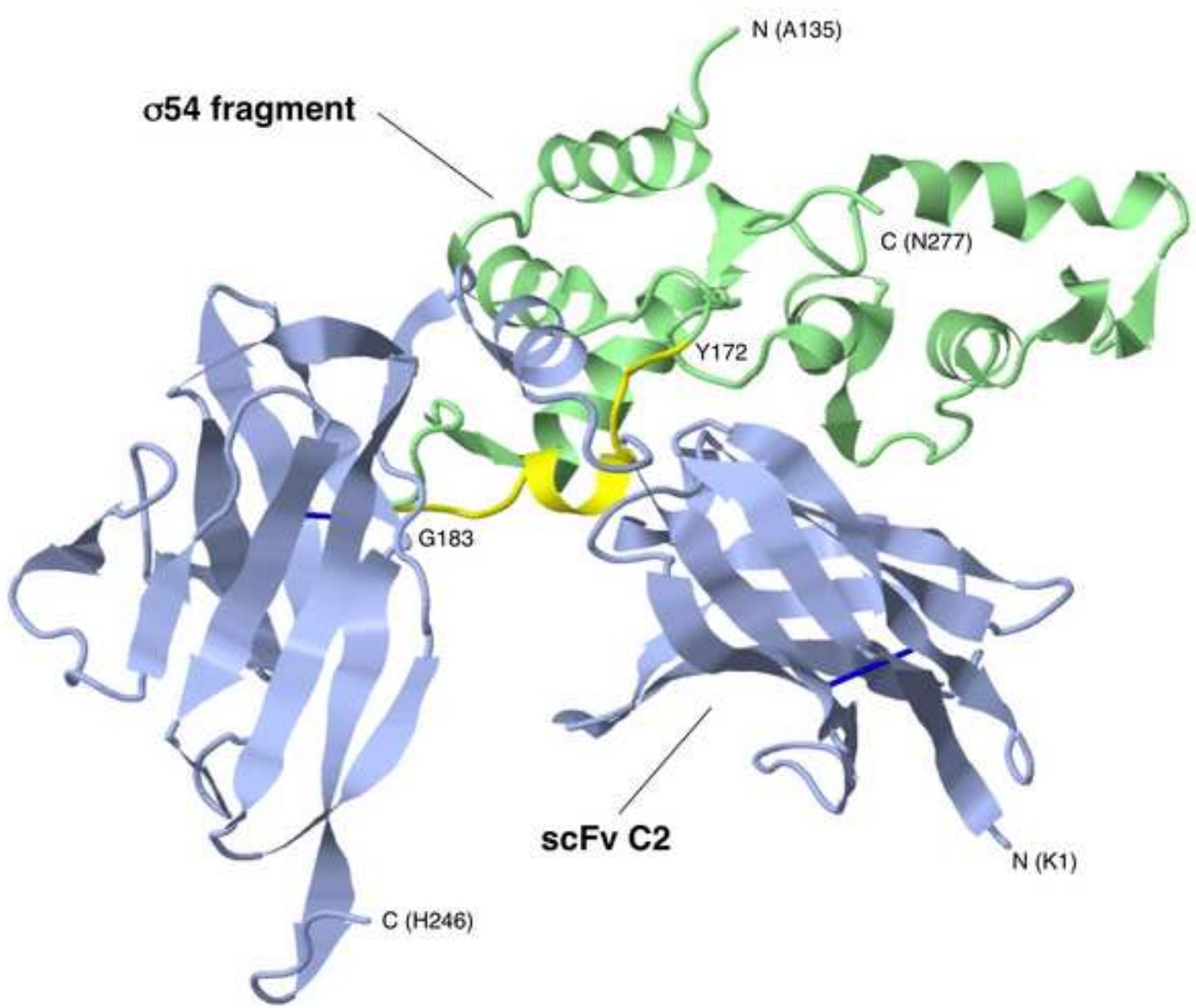

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