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Prokaryotic expression of antibodies and affibodies
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Recent advances have been made in the development of systems for the display and expression of recombinant antibodies and affibodies in filamentous phages, *Escherichia coli* and other prokaryotic cells. Emphasis has been placed on improving phage and phagemid vectors, alternative systems for expression in different cellular compartments (e.g., the outer membrane, periplasm, cytoplasm and extracellular secretion) and novel multimerization systems for generating bivalent or multivalent binding molecules.

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Abbreviations
Ab antibody
Af affibody
AnkR ankyrin repeat
CDR complementarity determining region
Fab antigen-binding fragment of antibodies
Ig immunoglobulin
MBP maltose-binding protein
rAb recombinant antibody
scFv single-chain antibody Fv fragment
sdAb single-domain antibody fragment

Introduction
The possibility of expressing antibodies (Abs) in large amounts and in clonal form in *Escherichia coli* cells has attracted the attention of biotechnologists since the early days of genetic engineering. However, production of complete immunoglobulins (Igs) turned out to be extremely difficult, given their structural complexity. This fact directed interest to the production of small Ab fragments that retain full antigen-binding capacity, a strategy that yielded the first successful reports of active Fab (antigen-binding fragment of antibodies composed of heterodimer VH–CH1/VL–CL) and single-chain Fv (scFv) fragments expressed in the periplasm of *E. coli* in the late 1980s. These studies were followed by the cloning of large repertoires of scFv and Fab genes in phage or phagemid vectors, allowing the display of these recombinant antibodies (rAbs) on the capsid of filamentous phage. Phage display permits the *in vitro* selection of clones with distinct antigen-binding specificities in a process named biopanning, which mimics the clonal expansion of B cells *in vivo* (Figure 1).

These initial findings triggered a research explosion in the field that has continued up to now. The generation of large combinatorial libraries of Fabs and scFvs, the engineering of selected clones to improve their binding and stability properties, and the design of new systems for their expression in different bacterial hosts, cellular compartments and protein formats (e.g., bivalent and multivalent molecules, diabodies, etc.) have been the major areas of investigation. The search for even smaller rAb fragments has led to the use of single-domain antibodies (sdAbs), based on natural V domains from heavy-chain-only Abs (e.g., VHH camelbodies) or engineered VH or VL domains with autonomous antigen-binding activity. Other antigen-binding fragments have been constructed using the rational design of binding capacities in small protein scaffolds, not based on Ig domains, and these are generally referred to as affibodies (Afs).

This review deals with the more recent developments in rAb and Af expression and display systems in prokaryotic cells. Given space limitations, structural studies addressing the interaction of rAbs and Afs with proteins and haptens will not be discussed [1–6]. The reader is also referred to general reviews for comprehensive coverage of this technology [7–14].

Phage display
rAbs and Afs are generally displayed in filamentous phages (e.g., M13) as fusions to the minor coat protein pIII (~3 to 5 copies/virion), which is essential for phage infection and packaging. Hence, vectors for phage display are either directly derived from complete phage genomes or are phagemids (plasmids with phage packaging signals) encoding pIII. Alternative phage-display systems have been reported, like those based on the minor coat protein pIX [15*], but have not been extensively used.

Choosing between a phage and a phagemid vector is relevant for biopanning and for the affinity of the selected clones. A recent study using a non-immune human scFv library [16] has clearly shown that phage vectors allow higher display levels and make biopanning more efficient (i.e. greater numbers of binders are isolated in fewer rounds). The reason for these differences stems from the need for a helper phage (e.g. VCS-M13 or...
M13KO7) for rescue of phagemid vectors (Figure 2). Wild-type pIII, encoded by the helper phage, is packaged more efficiently than scFv–pIII fusions encoded by phagemids. As a result, phagemid virions contain none or a single copy of the scFv–pIII hybrid, whereas several copies can be packaged in phage virions. In some situations, such as panning against rare targets on cell surfaces, phage multivalency is desirable. Other factors, such as culture conditions, E. coli strain, and the signal peptide present in the vector, also have an important influence on the display levels of pIII fusions [17,18]. In the case of Fabs, multivalency is difficult to achieve even with phage vectors owing to their larger size (proteolysis of the Fab fusion generates wild-type pIII that is packaged in the virion). A new vector system allows the display of bivalent Fabs fused to leucine zippers on phagemid virions [19].

By contrast, monovalency of phagemid vectors benefits the affinity of the selected scFv clones. On average, scFvs isolated from phagemids have five- to tenfold higher affinities than those from phage vectors, in which avidity effects allow the selection of low-affinity clones [16]. Panning with the antigen in solution can minimize the problems of low affinity associated with scFvs displayed on phage vectors [20].

A novel phagemid vector has been developed that enables the removal of phage particles not displaying an scFv–pIII fusion before panning [21]. The method is based on the production of fusions between scFv, a cellulose-binding domain (CBD) and pIII. Phage particles displaying scFv–CBD fused to pIII are captured on cellulose filters whereas ‘bald’ phages are removed by washing. An interesting option that combines the advantages of phage and phagemids systems is the use of mutant helper phages lacking pIII. The pIII mutant helper phages produce multivalent phagemid particles that can be used for the initial round of biopanning, whereas rescue with standard helper phages produces monovalent (high-affinity) particles for the following rounds [22]. Two improved mutant helper phages with partial deletions or amber stop codons in gene 3 have been reported (Hyperphage and Phaberge) [17,22]. Compared with other M13–pIII mutants [23,24], the new helpers appear to be more stable and produce higher titers of rescued phagemids. These mutant helper phages can also be extremely useful for the selective infection of phage (SIP) [25]. SIP phagemid vectors contain an N-terminal truncation of pIII that produces non-infective particles unless the displayed rAbs or Af s interact with the desired antigen (provided in trans and fused to the N-terminal domain of pIII) [26].
Bacterial display

The display of rAbs and Afs on the surface of bacteria is not only an alternative expression system for the screening of binders from libraries, but opens new potential applications — like the generation of whole-cell affinity sorbents, the delivery of passive immunity to mucosal body surfaces, and the targeting of bacteria to certain antigens or tissues. In E. coli, initial reports of the surface display of scFvs were achieved using lipoproteins [27] and lipoprotein–porin fusions (Lpp–OmpA) [28]. The major disadvantage of these expression systems is their toxicity for E. coli and the absence of a bona fide secretion of the scFvs, which become surface-exposed mostly because of the leakiness of the bacterial outer membrane after induction.

Recently, bacterial autotransporters have been proved as an effective system for the surface display of single Ig domains and stable scFvs in E. coli [29**]. Autotransporters comprise a large family of proteins secreted by Gram-negative bacteria. They are characterized by a C-terminal domain that inserts into the outer membrane and assembles into an oligomeric complex with a 2 nm hydrophilic pore through which the N-domain of the protein is translocated [30]. By substituting the natural protease N-domain of the IgA protease from Neisseria gonorrhoeae (an autotransporter) with different scFvs, V_{H\text{H}} domains, and strings of two or three V_{H\text{H}} domains, it has been shown that stable scFvs and V_{H\text{H}} can be displayed on the surface of E. coli cells with efficiencies close to 100% [29**]. Using this expression system it was also shown that E. coli cells displaying an scFv against enteric coronaviruses were able to act as delivery vehicles of passive immunity capable of neutralizing the infection of mammalian cells cultured in vitro [31].

A proof-of-principle of the delivery of passive immunity by commensal bacteria colonizing mucosal surfaces was obtained using an in vivo animal model and the food-grade Gram-positive Lactobacillus zeae displaying an scFv against the SAI/II adhesion molecule of Streptococcus mutans, the major pathogen involved in the development of dental caries [32**]. Efficient scFv display in L. zeae cells was achieved by fusion to the last 244 amino acid fragment of proteinase P. Rats that were orally treated with L. zeae cells displaying this scFv showed increased resistance to the development of dental caries.

Additional work has highlighted the potential use of Gram-positive microorganisms for the surface display of rAb and Af libraries. Afs based on the scaffold of protein A of Staphylococcus aureus have been displayed on Staphylococcus carnosus cells. Mixing an S. carnosus cell population displaying different Afs with fluorescence-labeled antigens allowed the selection of Afs with given
specificities by fluorescence-activated cell sorting [33]. The display system is based on the C-terminal cell-wall anchor domain of protein A of S. aureus [34].

**Expression systems for rAbs**

Figure 3 summarizes various strategies for targeting the expression of active rAbs to distinct compartments of *E. coli* (e.g. extracellular medium, outer membrane, periplasm and cytoplasm). Expression of rAbs is typically achieved by fusion to N-terminal signal peptides, which target the protein to the periplasmic space of *E. coli* where chaperones such as Skp, FkpA, DsbA and DsbC assist the folding of the Ig domains and form the correct disulfide bridges to stabilize the structure [35,36,37]. Production yields in the periplasm usually range from 0.1–10 mg/L of induced culture (OD₆₀₀nm = 1). *E. coli* host strains lacking the major periplasmic proteases (DegP and Prc) have been shown to increase the yield of Fab's produced in the periplasm two- to threefold [38]. In exceptional cases, much higher periplasmic yields of sdAbs have been reported (100 mg/L) [39].

Periplasmic overexpression may render an important fraction of the produced rAb insoluble. Fusion of scFvs to the periplasmic chaperones DsbC or DsbG, and co-expression of DsbC in *trans*, have been shown to increase significantly the fraction of soluble and functional scFv in the periplasm of *E. coli* [40].

Higher levels of rAbs can be produced in the cytoplasm of *E. coli* using common overexpression systems (e.g. T7 promoter vectors) and shake flask cultures (yields >50 mg/L/OD₆₀₀nm). However, these cytoplasmic rAbs are reduced (i.e. disulfide bonds are not formed in the cytoplasm of wild-type *E. coli* strains), unfolded, and form inclusion bodies that need to be solubilized under strong denaturing conditions (e.g. 8 M urea). The rAbs purified from these inclusion bodies can be refolded *in vitro* by dialysis of the denaturing agent in the presence of a redox pair (e.g. reduced and oxidized glutathione 1:1). The efficiency of refolding is highly variable depending on the specific clone, although excellent results have been reported for some scFvs [41,42].

An scFv can also be expressed in active (but not oxidized) form in the cytoplasm of wild-type *E. coli* cells by grafting its complementarity determining region (CDR) to frameworks regions derived from a highly stable scFv. In some cases, this process has been shown to maintain the unaltered specificity and affinity of the original scFv clone [43].

An alternative to the above approaches is the use of *E. coli* strains that promote the correct folding and oxidation of rAb in the cytoplasm *in vivo*. As oxidized scFvs seem to refold *in vitro* with higher efficiency [44], these strains may also be useful for the refolding of scFvs from...
inclusion bodies. Functional (correctly folded and oxidized) and soluble Fab and scFv molecules have been produced in the cytoplasm of *E. coli* cells carrying mutations in the genes coding for thioredoxin reductase (trxB) and glutathione oxidoreductase (gor), with yields similar or even higher than those obtained in the periplasm [37**,45**,46**]. *E. coli trxB gor* mutant cells have an oxidizing cytoplasm capable of forming disulfide bridges in proteins [47,48]. At induction temperatures above 30°C efficient folding of Fabs and scFvs in *E. coli trxB gor* mutants appears to require cytoplasmic co-expression of the periplasmic chaperones DsbC or Skp (devoid of their N-terminal signal peptides) [37**,45**]. Interestingly, in *vivo* biotinylation of an scFv expressed in the absence of chaperones in the cytoplasm of *E. coli trxB gor* cells was only efficient at temperatures below 30°C [49*]. Biotinylated rAbs can be bound to avidin- and streptavidin-containing resins (e.g. streptavidin magnetic beads) and immunoconjugates (e.g. streptavidin-peroxidase) for purification, immunoprecipitation and detection purposes.

Protein chaperones are not always needed. Two Fabs were shown to accumulate at high levels in active form in the cytoplasm of *E. coli trxB gor* cells (10–30 mg/L OD600nm) in the absence of chaperones [46**]. Similarly, a catalytic scFv fused to the C terminus of NusA was produced in a folded form in the cytoplasm of *E. coli trxB gor* cells without co-expression of chaperones [50]. Significantly, the unfused scFv aggregates in inclusion bodies in the cytoplasm of *E. coli trxB gor* cells and was rapidly degraded in wild-type *E. coli* cells, either alone or fused to NusA. N-terminal fusions to maltose-binding protein were also shown to improve the expression of scFvs in the cytoplasm of wild-type *E. coli* cells (but in a reduced and only partially folded form) [51]. Taken together, the above data indicate that folding and oxidation of rAbs generally requires the cytoplasm of *E. coli trxB gor* cells and the activity of chaperones (e.g. DsbC) or other solubilizing factors (e.g. N-terminal fusions), although particular clones may fold efficiently in their absence.

An alternative to the periplasmic and cytoplasmic expression of rAbs is their secretion to culture supernatants using the z-hemolysin (HlyA) system of *E. coli* [52**,53**]. HlyA is a monomeric toxin that is secreted directly from the cytoplasm into the extracellular medium across a three-component protein channel (TolC/HlyB/HlyD) connecting the inner and outer membrane [54,55]. scFvs and sdAbs, devoid of N-terminal signal peptide and fused to the C-terminal domain of HlyA, have been secreted into the culture medium by *E. coli* cells expressing TolC/HlyB/HlyD. These rAbs accumulated as the sole polypeptide in the culture medium at concentrations similar to those obtained by their periplasmic expression (0.5–2 mg/L). The mechanism of folding and oxidation of rAbs secreted by the HlyA pathway is unclear, but appears to be intimately associated with the TolC/HlyB/HlyD channel and is independent of periplasmic chaperones (e.g. DsbA, DsbC) [56]. As different rAbs have distinct folding requirements, it remains to be determined to what extent this periplasmic-independent pathway is compatible with the folding of the diverse sequences found in rAb libraries.

**Oligomerization strategies**

The oligomerization of rAbs to make bivalent and multivalent molecules of high functional affinity (avidity) has been elicited using a variety of approaches. Earliest reports employed dimerizing and oligomerizing protein motifs (e.g. amphipathic helices) with and without stabilizing disulfide bonds (reviewed in [13]). Alternatively, shortening the length of the linker peptide connecting the VH-VL domains in scFvs generates diabodies (scFv dimers), triabodies and tetrabodies, with mono- bi- tri- or tetra-antigen specificities (reviewed in [57]). Bivalent dimeric and bi-specific camel sdAbs have also been produced by fusing two VHH domains with a natural hinge peptide [58]. Figure 4 summarizes the structure of various rAbs (Fabs, scFvs and sdAbs) and some of the oligomeric forms that are discussed here.

In general, rAbs of high avidity can be obtained with the above techniques, although they suffer some problems related to aggregation, unintended swapping between V domains, and instability in the bloodstream. Therefore, current studies are focusing on the major factors influencing their stability and expression [59,60], while others search for new methodologies to produce oligomeric rAbs of high-avidity. For instance, novel ‘di-diabody’ molecules of high stability have been obtained in the periplasm by the interaction of two bi-specific diabody molecules with the constant C1H3 domain from a natural IgG [61]. Stable dimers of VHH have been secreted to the supernatants of *E. coli* cultures using a modified HlyA signal containing the leucine zipper of GCN4 [52**]. Another report used the B subunit of the Verotoxin 1 (VT1B), a Shiga-like A:B5 toxin from *E. coli* O157:H7, to assemble stable and soluble pentamers of sdAb with high avidity in the periplasm [39,62**]. The sdAb molecules were derived from a naïve llama VHH library and the small VT1B monomer (7.7 kDa) was fused to the N termini of the sdAbs, which did not interfere with their binding properties.

A novel multimerization system, based on the interaction between the prokaryotic ribonuclease Barnase (110 amino acids) and its inhibitor Barstar (89 amino acids), has revealed important advantages [44**]. Barnase and Barstar interact with extremely high affinity (KD ~10−14 M), thus making complexes of remarkable stability. These polypeptides are soluble, fold independently of its interacting pair, and can be secreted to the periplasm. Fusing these proteins to the C terminus of scFvs has allowed the production of stable mono-, di- and trivalent complexes.
of high avidity and with long half-lives in vivo. Of specific note using this approach, homogeneous bi-specific dimers can be produced in vitro during the process of large-scale protein purification by in-column refolding.

**Protein modifications**

Various modifications have been engineered into rAbs and Afs to assist downstream processing. For instance, scFvs fused to two chitin-binding domains can be immobilized on inexpensive chitin beads directly from crude protein extracts and used in immunoaffinity chromatography to purify proteins recognized by their scFv moiety [63]. Fusion of one or two Afs (based on the 58 amino acid three-helix bundle domain of staphylococcal protein A) to β-galactosidase (a homotetramer in the cytoplasm of E. coli) resulted in immunoconjugates that exhibited improved binding properties [64,65]. The (Af2-β-galactosidase)₄ complex was produced at high yields in shake flask cultures of E. coli (~400 mg/L) and could be used directly in enzyme-linked immunosorbent assays and immunohistochemistry [64*].

Phage display vectors have also been modified to produce scFvs with an extra cysteine residue near their C termini [66]. The free thiol group of this cysteine allows the specific chemical crosslinking of polyethylene glycol (PEG) maleimide to the rAb. The covalent attachment of PEG (PEGylation) can extend plasma half-life and increase the solubility of rAb and other proteins [67]. Another important protein modification is biotinylation. scFvs can be biotinylated in vivo in the cytoplasm of E. coli trxB gor cells with very high efficiencies (>90% of the total scFv produced) [49]. This has been achieved by fusion of the scFv to the 89 amino acid C-domain of the VT1B.
bifunctional proteins, in which one function is an enzyme or a ligase and the other is a reporter molecule. For instance, a library of sdAbs based on heavy-chain-only Abs of the isotype novel antigen receptor (IgNAR) was obtained from nurse sharks (Ginglymostoma cirratum) immunized with hen egg-white lysozyme (HEL). From this library, sdAb binders of high stability and with high affinity to HEL have been selected by phage display [68].

Two Af libraries have been constructed based on ankyrin repeats (AnkR) as protein scaffolds [69**]. AnkR proteins are composed of several 33 amino acid repeats stacked in a row, each repeat comprising a β-turn followed by two antiparallel α helices and a C-terminal loop [71]. The AnkR proteins are found in organisms from all phyla and can be located in the cytoplasm, anchored to membranes or secreted to the extracellular space. The strategy to generate the AnkR Af libraries was based on the design of a synthetic AnkR consensus sequence, derived from natural AnkR sequences, with randomized amino acid positions at the β-turn and the short hinge connecting the two α helices. This AnkR module was repeated two or three times, depending on the specific library, and the fusion proteins were flanked at their N and C termini with capping AnkR of defined sequence [69**]. The AnkR AfS were highly stable, soluble and accumulated in the cytoplasm of E. coli at high concentrations (yields averaged 200 mg/L of culture in shake flasks). More importantly, high-affinity binders (Kd in the nanomolar range) against different protein targets (e.g. MBP) were selected from these libraries using ribosomal display (a powerful technique for the selection of binders directly from an in vitro transcription-translation reaction [72,73]). The same report also showed a co-crystal of MBP with its binder AnkR Af, revealing the interaction surface between these two molecules. The three-dimensional X-ray structure of the MBP–Af complex showed the randomized amino acids of the AnkRs forming a concave surface contacting MBP and covering ~600 Å² of its surface, which is only slightly smaller than the surface covered in antigen–antibody complexes (777 ± 135 Å²) [74]. Given the extraordinary properties of AnkR AfS (e.g. high solubility, affinity, flexible modularity, absence of disulfide bonds, etc.), these AfS may be suited not only for in vitro applications (e.g. affinity purification, protein co-crystallization and protein chips), but might also have a role as intracellular inhibitors of specific processes in vivo.

Finally, a recent report [75] described the generation of a new Af library based on a stable variant of green fluorescent protein (GFP), although this has not been confirmed [76].

Conclusions
Although additional work is still needed to increase the functional expression of rAbs and AfS in various cellular compartments of E. coli, this organism is clearly the best choice for these technologies given its high transformation efficiencies and the panoply of vector systems to display, express and modify rAbs and AfS. Future research is likely to improve functional expression, select new stable protein scaffolds with desired properties for specialized applications, integrate affinity maturation and display systems, and expand the use of these technologies to other prokaryotic microorganisms.

Update
A recent study evaluates the influence of different vector and culture conditions for efficient Fab phage display and expression in E. coli [78]. The S-layer protein of Bacillus sphaericus CCM 2177 has been fused at its C terminus to a V₁₁₁ camel antibody against prostate cancer specific antigen (PSA). The hybrid S-layer protein retained the ability to self-assemble with the V₁₁₁ moiety exposed to the outer surface of the protein lattice. After recrystallization on gold chips, this protein lattice was used as a sensing layer in surface plasmon resonance to detect PSA [79].

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