Production and fluorescence-activated cell sorting of Escherichia coli expressing a functional antibody fragment on the external surface

JOSEPH A. FRANCISCO*, ROB CAMPBELL†, BRENT L. IVERSON‡, AND GEORGE GEORGIOU§

Departments * of Chemical Engineering and †Chemistry and Biochemistry, The University of Texas, Austin, TX 78712; and ‡Becton Dickinson Research Center, P.O. Box 12016, Research Triangle Park, NC 27709-2076

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ABSTRACT We have expressed a single chain Fv (scFv) antibody fragment, consisting of the variable heavy and variable light domains from two separate anti-digoxin monoclonal antibodies, on the external surface of Escherichia coli by fusing it to an Lpp–OmpA hybrid previously shown to direct heterologous proteins to the cell surface. This scFv fusion was expressed at a high level and was shown to bind the hapten with high affinity and specificity. Whole cell ELISAs, fluorescence microscopy, protease sensitivity, and flow cytometry all confirmed that the scFv was anchored on the outer membrane and was accessible on the surface. Utilizing fluorescence-activated cell sorting, we were able to specifically enrich scFv-producing cells from a 105-fold excess of control cells in only two steps. The expression of antibody fragments on the surface of E. coli is being evaluated as an attractive method for the in vitro production and selection of useful antibody fragments.

A general method for the identification of protein sequences possessing a high affinity for a particular ligand can greatly facilitate a better understanding of biomolecular recognition and will have important applications in fields such as drug design and antibody engineering. One of the most successful approaches for identifying proteins having a desired affinity is by screening large libraries generated by genetic means (1–4). A widely used technique for screening such libraries is based on the display of proteins or peptide sequences on the surface of filamentous phage (1, 2). Briefly, recombinant proteins are displayed on the surface of phage particles as fusions to the N terminus of either gIII or gVIII coat proteins. Subsequently, the library of phage clones is screened for binding to the target molecule attached to a solid phase. Successive rounds of binding and elution result in selective enrichment of phage possessing proteins or peptide sequences that are specific for the target molecule. However, the binding to the target immobilized on a solid phase depends not only on molecular affinity but also on other factors such as surface characteristics, number of attachment points (avidity effects), and hydrodynamic conditions.

Libraries of antibody molecules expressed as single chain Fv (scFv) or Fab fragments have been displayed on the surface of phage and screened for binding toward immobilized antigen (5). Several recent studies with phage have demonstrated that the in vitro screening of antibody libraries represents a promising method for the isolation of interesting antibody fragments specific for a variety of different molecules. Libraries have been constructed from the PCR products derived from antibody-producing cells isolated from animals or through a semisynthetic strategy involving the randomization of antibody complementarity-determining regions using synthetic oligonucleotides (6–12). This latter approach is especially interesting as a way to expand the immunological repertoire beyond what is found in nature.

In theory, the cell-associated fluorescence due to the binding of a fluorescent hapten could be employed to select Escherichia coli displaying surface-anchored antibody fragments from a large excess of negative cells (13). Along these lines, a previous attempt at placing an active antibody fragment on the surface of E. coli has been reported (14, 15). However, this construct was not fully characterized or used for any selection experiments.

Herein, we report the production and characterization of a functional scFv antibody fragment attached to the outer surface of E. coli. Using a fluorescently labeled hapten, we have found that these constructs can be efficiently selected using flow cytometry. A 105-fold enrichment was reproducibly obtained after only two rounds of sorting followed by growth of the sorted cell population. Our E. coli surface expression and flow cytometry selection system is being investigated as a “user friendly” technology for the production and isolation of interesting antibody fragments in vitro.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. E. coli strain JM109 (endA1 recA1 gyrA thi-1 hsdR17 (rK-, mK+ relA supE44 Δ(lac-proAB)/F’ traD36 proAB lacF’ lacZDΔM15) was used for all experiments. pTX101 codes for an Lpp–OmpA–β-lactamase fusion (16). pTX152 codes for an Lpp–OmpA–scFv(digoxin) fusion, where the scFv(digoxin) is an anti-digoxin scFv consisting of the heavy- and light-chain variable regions (VH and VL). The VH and VL, joined by a 15-amino acid (Gly)5Ser5 linker (17), were amplified from mRNA isolated from two separate anti-digoxin hybridomas. An 11-amino acid peptide from the herpes simplex virus (HSV) glycoprotein (Novagen) was introduced at the C terminus of the scFv for analytical purposes. To construct pTX152 the bla from pTX101 was first removed by digestion with EcoRI and BamHI. Subsequently, the amplified gene coding for the anti-digoxin scFv was digested with EcoRI and BamHI and ligated into pTX101. Both pTX101 and pTX152 carry the chloramphenicol-resistance gene.

 Cultures were grown in LB medium (Difco) supplemented with 0.2% glucose and chloramphenicol (50 μg/ml).

ELISA. Overnight cultures grown at 24°C were harvested, resuspended in phosphate-buffered saline (PBS) at OD600 = 2.0, and lysed by passage through a French pressure cell at 20,000 psi (1 psi = 6.89 kPa). The lysates were then diluted with 1 volume of PBS containing 2.0% bovine serum albumin (PBS/2% BSA) and 5 mM of the protease inhibitor phenyl-

Abbreviations: scFv, single chain Fv; VH, heavy-chain variable region; VL, light-chain variable region; BSA, bovine serumalbumin; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HSV, herpes simplex virus.

*To whom reprint requests should be addressed.
methylsulfonyl fluoride. Microtiter plates (96 wells) were 
incubated overnight at 37°C with 100 μl of 100 μg/ml of either 
BSA or digoxin-conjugated BSA (digoxin-BSA) in 0.1 M 
sodium carbonate buffer (pH 9.2). All subsequent steps were 
carried out at room temperature. The wells were fixed for 5 
min with 100 μl of methanol and were then blocked for 45 min 
with 200 μl of PBS/1% BSA. After removing the blocking 
solution, the wells were incubated for 2 hr with 100 μl of 
lyses. Subsequently, they were washed three times with 
200 μl of PBS/0.1% Tween 20 and incubated for 1 hr with 100 μl 
per well of monoclonal antibodies against the HSV peptide 
or antiserum against β-lactamase. The wells were again 
washed three times with PBS/0.1% Tween, incubated for 1 
hr with 100 μl of the appropriate secondary antibodies 
coupled with horseradish peroxidase, and finally washed 
five times with PBS/0.1% Tween and two times with PBS. 
After addition of the substrate 2,2'-azinobis(3-ethylbenzthi-
azoline-6-sulfonic acid) (Pierce) the absorbance of each well 
was measured at 410 nm.

Whole cell ELISAs were performed as described above 
extcept that 100-μl samples of overnight cultures that had 
been resuspended in PBS/1% BSA at OD₆₀₀ = 1.0 were used 
instead of cell lysates.

**Fluorescence Microscopy and Fluorescence-Activated Cell 
Sorting (FACS).** For fluorescence microscopy, overnight 
cultures grown at 24°C were harvested, resuspended at OD₆₀₀ = 
0.5 in PBS containing 10⁻⁷ M fluorescein-conjugated 
digoxin (digoxin-FITC; ILS LTD, London), and incubated 
at room temperature for 1 hr. Prior to microscopy the cells were 
washed once with PBS and resuspended in equal volumes of 
PBS and Vectashield mounting medium (Vector Laborato-
ries) at OD₆₀₀ of ≈ 2.0.

For the FACS experiments, overnight cultures grown at 
24°C were harvested and resuspended in PBS at OD₆₀₀ = 0.5. 
Positive (JM109/pTX152) and control (JM109/pTX101) cells 
were mixed at the desired ratios and the mixture was 
incubated at room temperature for 1 hr with 10⁻⁷ M digoxin-
FITC. The cells were then pelleted by centrifugation, 
resuspended in PBS at 3 × 10⁶ cells per ml, and either counted 
or sorted on the basis of fluorescence intensity using a FACS 
fluorescence-activated cell sorter (Becton Dickinson). Flow 
rates of between 12 and 60 μl of sample per minute were used, 
with 488-nm excitation (argon-ion laser) and emission detection 
between 515 and 545 nm. Counting runs involved at least 10,000 
cell events per experiment. The sorting experiments in-
involved 100,000–500,000 cells per run. After sorting, the 
selected cells were added to 50 ml of LB broth containing 50 
μg of chloramphenicol per ml and 0.2% glucose and then 
grown overnight at 37°C with shaking. Subsequently, the 
cells were subcultured into fresh medium and grown over-
night at 24°C with shaking. Finally, the culture was incubated 
for 1 hr with 10⁻⁷ M digoxin-FITC, resuspended in PBS at 3 
× 10⁶ cells per ml, and run through another round of FACS.

**General Procedures.** The protein composition of whole 
cell membrane fractions isolated from overnight cultures was 
analyzed by SDS/PAGE on 12% acrylamide gels and by 
Western blotting (18) using anti-HSV monoclonal antibodies 
(Novagen) and anti-OMP A antiserum. Whole membrane frac-
tions were prepared as described (16).

### RESULTS AND DISCUSSION

**Expression of Lpp−OMP(A₄₋₁₅₉)−scFv(digoxin).** The cell 
envelope of *E. coli* and other Gram-negative bacteria consists 
of the inner membrane (cytoplasmic membrane), the pepti-
doglycan cell wall, and the outer membrane. Although the 
latter normally serves as a barrier to protein secretion, we 
have constructed a chimeric targeting sequence that, when 
fused to normally soluble proteins, can direct them to the 
cell surface (16). The chimeric targeting sequence consists of the 
leader peptide and first 9 amino acids of the *E. coli* major 
outer membrane lipoprotein (Lpp) and amino acids 46–159 of 
the outer membrane protein OmpA. The function of the Lpp 
is to direct the chimera to the outer membrane. Laukkanen 
et al. (19) have recently demonstrated that an active scFv 
antibody fragment can be anchored to the internal face of the 
*E. coli* outer membrane when fused to this region of Lpp. The 
OmpA region in our chimeric targeting sequence then 
switches the membrane leaving its C terminus exposed on the 
cell's exterior. We have used Lpp−OmpA(A₄₋₁₅₉) fusions to 
anchor a variety of proteins such as β-lactamase, a cellulose 
binding protein, and alkaline phosphatase on the *E. coli* 
surface (refs. 16 and 20; C. Stathopoulos, C. F. Earhart 
and G.G., unpublished data).

For the display of antibodies on the surface of *E. coli* a scFv 
specific for digoxin was constructed as described in *Materials 
and Methods*. For detection purposes, a 33-bp fragment 
encoding an epitope from the HSV glycoprotein was added to 
the 3' end of the *V₅* to facilitate detection by immunochem-
ical techniques. The nucleotide sequence of the antibody 
fragment is shown in Fig. 1. The Lpp−OmpA(A₄₋₁₅₉)− 
scFv(digoxin) construct is encoded by the plasmid pTX152. 
*L. coli* JM109 was transformed with pTX152, grown at 37°C, 
and used to isolate total membranes. In Western blots a band 
of the expected size (42 kDa) was recognized by antibodies 
specific for both OmpA and the HSV peptide (Fig. 2A). The 
near absence of lower molecular mass bands crossreacting 
with either the anti-HSV or the anti-OmpA antibodies indi-
cates that the scFv(digoxin) was not subjected to proteolysis, 
probably because it is anchored on the cell surface (see 
below), and consequently it is physically separated from 
intracellular proteases. The intensity of the Lpp−OmpA(A₄₋ 
1₅₉)−scFv(digoxin) band in Fig. 2A is comparable to that of 
the native OmpA band. The latter is a highly expressed 
protein that is present in the *E. coli* outer membrane at 
about 100,000 copies per cell (21). Thus the level of expression 
of Lpp−OmpA(A₄₋₁₅₉)−scFv(digoxin) appears to be on the 
order of 50,000–100,000 copies per cell.

**Display of scFv(digoxin) on the Cell Surface.** The ability of 
the scFv domain of the fusion protein to bind the hapten was 
determined by ELISA. Whole cell lysates from JM109/ 
pTX152 and JM109/pTX101 were incubated on microtiter 
plates that had been coated with either digoxin-conjugated 
BSA or digoxin-BSA) or unconjugated BSA. Subsequently, 
the wells were treated with antibodies against the HSV peptide 
or β-lactamase as necessary to detect the respective fusion 
proteins. JM109/pTX152 lysates bound specifically to wells 
coated with digoxin-BSA but not to unconjugated BSA, 
whereas the lysates from the control strain, JM109/pTX150, 
did not give a signal with either. Thus, Lpp−OmpA(A₄₋₁₅₉)− 
scFv(digoxin) is active and can bind to the hapten specifi-
cally.

Fig. 2B shows the results of ELISAs using intact cells. 
Samples containing the same number of cells were used in 
all experiments described here. Cells containing pTX101 gave 
the same low signal when incubated on microtiter wells 
coated either with unconjugated BSA or with digoxin-BSA. 
A similar weak signal was detected with JM109/pTX152 
incubated on BSA-coated wells and is presumably due to 
non-specific binding. In contrast, a much higher absorbance 
was evident in wells coated with the digoxin-BSA, indicating 
that there are active fusion protein molecules on the cell 
surface.

The display of the active scFv antibody on the cell surface 
was confirmed by fluorescence microscopy (Fig. 3). JM109/ 
pTX152 cells were grown overnight at 24°C, incubated with 
10⁻⁷ M digoxin-FITC for 1 hr, and washed. As shown in Fig. 
3, all of the cells visible with phase-contrast microscopy gave 
a strong fluorescence signal. In control experiments, when 
JM109/pTX101 cells were incubated with the same concen-
tration of digoxin-FITC and then washed, none of the cells became fluorescently labeled (data not shown). Furthermore, protease treatment drastically reduced the ability of the cells to bind the fluorescently labeled hapten, as evidenced both by fluorescence microscopy and by FACS (Fig. 4C).

The intensity of the fluorescence signal from JM109/pTX152 was dependent on the cell growth temperature and was much higher for cultures grown at 24°C instead of 37°C. This is consistent with previous results that show that the amount of proteins expressed on the surface of E. coli by fusion to Lpp-OmpA(46–159) increases as the temperature is decreased (16, 20). If the efficiency of surface display in this case is similar to that of β-lactamase (16), then at 24°C virtually all the scFv antibody chains should be accessible on the cell surface.

Detection by FACS. Before FACS could be performed it was first necessary to determine whether E. coli displaying the scFv(digoxin) antibody and labeled with digoxin-FITC could be discriminated from the background. Samples of 10^8 cells per ml from cultures grown at 24°C were incubated with digoxin-FITC at 10^{-7} M, washed in buffer, and diluted to 3 × 10^6 cells per ml prior to sorting. The samples were then analyzed using a FACSsort flow cytometer. Fig. 4A and B show that the fluorescence intensity of JM101/pTX152 was clearly distinguishable from the intrinsic background signal of control E. coli (in this case, JM101/pTX101). When JM109/pTX152 cells were preincubated, with an excess of free digoxin prior to incubation with the digoxin-FITC, the fluorescence intensity of the cells was the same as for the background (Fig. 4D). This specific inhibition was also seen using fluorescence microscopy and demonstrates that the surface-expressed scFv(digoxin) specifically binds the fluorescently labeled hapten in the binding site and is not the result of nonspecific interactions.

Treatment of intact cells with trypsin prior to incubation with digoxin-FITC almost completely eliminated the population of fluorescently labeled cells detected by flow cytometry (Fig. 4C). In Gram-negative bacteria, the outer membrane serves as a barrier to preclude the diffusion of large extracellular molecules such as proteins. The action of trypsin is assumed to be limited to the proteolysis of proteins exposed on the external surface of E. coli (22). As such, the above result provides further evidence that the active scFv-(digoxin) is indeed accessible on the outer surface, free to interact with molecules in solution.

The high background due to light scattering precluded a detailed quantitative evaluation of the K_d for the surface-expressed scFv(digoxin) using cell sorting of samples incubated with different concentrations of digoxin-FITC. However, the scFv(digoxin) binding sites appeared to be fully saturated at concentrations of digoxin-FITC above 10^{-7} M. Appreciable fluorescent signal was clearly detected at digoxin-FITC concentrations of 10^{-9} M. These results are consistent with a binding constant that is at least within an order of magnitude of the value of 1 × 10^{-9} M^{-1} determined for the scFv(digoxin) in solution (R.C. and P. Hamilton, unpublished data).

Enrichment of Cells Displaying scFv(digoxin) by FACS. We found that antibody-expressing cells can be sorted essentially quantitatively from a moderate excess of control E. coli in a single step. Specifically, in mixtures containing JM109/pTX101 at an excess, over JM109/pTX152, of either 100:1 or 1000:1, the fraction of the total population that was sorted in the high fluorescence intensity window was 1.1% and 0.1%, respectively, as expected from the ratio of input cells (data not shown). To evaluate the potential use of FACS for isolating rare clones from a very large excess of background, JM109/pTX101 and JM101/pTX152 were mixed at a ratio of 100,000:1 and labeled with digoxin-FITC; 500,000 cells from the input mixture were run through the FACSort flow cytometer. In this experiment a wide sorting gate—i.e., the minimum fluorescence required for acceptance of an individual cell—was selected such that up to 0.2% of the control cells fell within the sorting window. This ensured that all scFv(digoxin)-expressing cells would be recovered. The cells having an allowable fluorescence signal were collected and grown in fresh medium. Following growth, the cells were again run through the FACSort, grown in fresh medium as before, and resorted. To ensure the complete absence of artifacts due to nonspecific cell adhesion in the flow path of
Fig. 2. (A) Western blot of total membrane fractions from JM109/pTX101 (lanes 2 and 4) and JM109/pTX152 (lanes 3 and 5). Lanes 2 and 3 were probed with anti-OmpA antiserum at 1:5000 dilution. Lanes 4 and 5 were probed with monoclonal anti-HSV antibodies at 1:5000 dilution. Arrowheads indicate the Lpp-OmpA-β-lactamase fusion (lane 2) and the Lpp-OmpA-scFv(digoxin) fusion (lane 3). The 32-kDa band in lanes 2 and 3 corresponds to OmpA. Lane 1, molecular mass markers (in kDa). (B) Lysate and whole cell ELISAs of JM109 cells containing plasmid pTX101 (n) or pTX152 (o). Samples were incubated on microtiter wells coated with digoxin-conjugated BSA and probed with anti-β-lactamase (pTX101) or anti-HSV (pTX152) antibodies. Absorbance readings were referenced to wells that were untreated with either lysates or whole cells.

Fig. 3. Phase-contrast (A) and fluorescence (B) micrographs of the same field of JM109/pTX152 cells after 1 hr of incubation with 10−7 M digoxin-FITC.

the FACSsort, each run was followed by extensive washing with bleach. Fig. 4 E–G show the cell fluorescence distribution for the sorting runs. After only two rounds of growth and sorting, the fluorescence intensity of 79% of the cell population fell within the positive window. A similar enrichment was reproducibly obtained in three independent experiments. Importantly, these results are not due to a growth advantage of the cells expressing Lpp-OmpA(46–159)–scFv(digoxin), since successive regrowth of the input cell mixture in the absence of sorting did not result in any detectable enrichment.

To verify that the cells with the increased fluorescent signal after the final sorting step were indeed JM109/pTX152, we took advantage of the fact that pTX152 confers resistance only to chloramphenicol, whereas the plasmid present in the control cells also confers resistance to ampicillin. A sample of cells from the final round of FACS was plated on chloramphenicol plates and then replica plated on plates containing 100 μg of ampicillin per ml. Over 95% of all the colonies examined were chloramphenicol resistant/ampicillin sensitive (cm+/amp−), consistent with the phenotype expected for JM101/pTX152. As an additional test, plasmid DNA was isolated from eight cm+/amp− colonies and the presence of pTX152 was confirmed by restriction analysis.

Conclusions. We have demonstrated that a scFv antibody fragment specific for digoxin can be expressed on the E. coli
surface and binds to the hapten with high affinity. Using FACS, the antibody-expressing cells were recovered from a 10^3-fold excess of control E. coli in only two rounds of sorting and regrouping of the sorted cell population (Fig. 4). Sorting is both rapid and efficient. Using a low-end flow cytometer it is possible to sort 1 x 10^6 cells per hour when operated so that the cells pass through the laser beam single file. Higher sorting rates can be obtained with a larger model (up to 5 x 10^7 cells per hour) and even higher still by sorting multiple cells at a time (at the expense of increased background that can presumably be eliminated by resorting the selected population). In addition, the sorting of positive clones is essentially quantitative and is limited only by the accuracy of the flow cytometer, which is on the order of 95% (23).

Preliminary experiments have been undertaken using expression on the E. coli surface and flow cytometry to select for high-affinity antibodies from libraries derived from immunized animals as well as from a semisynthetic library and also screening peptide libraries for sequences with high affinity for various fluorescently labeled receptors. It is anticipated that by using very low concentrations of fluorescently labeled hapten or receptor (i.e., <10^-8 M), it should be possible to select directly for antibodies or peptides with very high affinity (i.e., K_a > 10^8 M^-1). In our system avidity effects due to multivalent binding do not come into play in the selection of high-affinity clones. In contrast, the high number of scFv (10^6 molecules or higher) on the cell surface should facilitate fine affinity discriminations. These characteristics, combined with the simple nature of the techniques involved, could make our E. coli-based system an attractive alternative to phage display for the isolation of high-affinity molecules from large libraries.

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