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Growth at sub-optimal temperatures allows the production of functional, antigen-binding Fab fragments in *Escherichia coli*

(Carcinoembryonic antigen; inclusion bodies; recombinant antibody; recombinant DNA)

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SUMMARY

Expression in *Escherichia coli* of recombinant genes coding for the κ-chain and the Fd fragment of an antibody directed against carcinoembryonic antigen gives rise to Fab dimers. These Fab fragments possess antibody activity, as demonstrated by enzyme-linked immunosorbent assay as well as by ligand competition assay. Effective production of soluble Fab in *Escherichia coli* was achieved by a decrease in the growth temperature. Following a one step purification by anion exchange chromatography, the bacterially-produced Fab retains its activity at 4°C for at least two months. The relatively simple methodology described in this study should be useful for the design and production of antibodies in bacteria.

INTRODUCTION

Genetic engineering provides a means for the manipulation of antibody (Ab) structure and specificity. Several forms of recombinant Abs have been constructed: human/mouse chimeric Abs (Boulianne et al., 1984), enzyme-linked Fab fragments (Neuberger et al., 1984), Fv fragments (Skerra and Fluckthun, 1988) and 'single chain antibodies' (Bird et al., 1988; Huston et al., 1988). Recombinant Ab genes have been expressed in *Escherichia coli* (Boss et al., 1984; Cabilly et al., 1984), in yeast (Horwitz et al., 1988) and in mammalian lymphoid cells (Neuberger et al., 1984). Even though lymphocytes are naturally adapted for Ab production, bacteria have potential advantages in terms of recombinant gene manipulations, growth, handling and economy. However, immunoglobulin (Ig) polypeptides precipitate in the bacterial cytoplasm as inclusion bodies, and solubilization of these bodies

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Abbreviations: Ab, antibody; Ag, antigen; B-Fab, bacterially-produced Fab; BSA, bovine serum albumin; CEA, carcinoembryonic antigen; DE-52, diethylaminoethyl-cellulose; EIA, enzyme immunoassay; Fab, antigen-binding fragment; Fd, truncated heavy chain; *Fd'*, gene encoding Fd; Ig, immunoglobulin; *k*, gene encoding κ chain; Fv, variable region fragment; PBS-NP40, 0.15 M NaCl/0.01 M Na·phosphate pH 7.2/0.05% (v/v) Nonidet-P40; PMSF, phenylmethylsulfonyl fluoride; RBS, ribosome-binding site; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TS buffer, 10 mM Tris-HCl pH 8/150 mM NaCl/0.05% (v/v) Nonidet-P40; v region, variable region.

has required the use of chaotrophic agents followed by renaturation procedures. These procedures give very poor yields of Ab activity (Boss et al., 1984; Cabilly et al., 1984). Recently it was reported that the introduction of a bacterial signal sequence into Ig-encoding genes promotes the secretion of active Fab or Fv fragments (Better et al., 1988; Skerra and Fluckthun, 1988).

I here report that in *E. coli* cells growing at 21°C, a single expression plasmid coding for κ-chains and truncated heavy chains (Fd fragments) gives rise to high yields of functional Fab fragments.

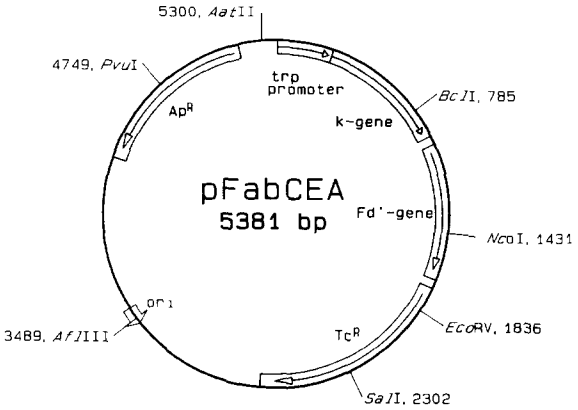

Fig. 1. Structure of the κ and Fd expression vector pFabCEA. Two previously described expression plasmids (Cabilly et al., 1984), pkCEAtrp 207-1* and pγFd′CEAtrp 207-1* were used to construct a single transcription unit gene encoding the κ-chain (*k*) and the Fd-fragment (*Fd′*). A *HpaI-SalI* fragment which contains the *Fd* gene was isolated from the γFd′CEA 207-1* plasmid and inserted into the κ-chain expression vector pkCEAtrp 207-1*. The latter vector was linearized with *Eco*RI, followed by filling in 5′ overhangs using Klenow polymerase and digestion with *SalI*. That way, the *k* and *Fd′* genes were successively positioned under the control of the *trp* promoter. Each of these genes is preceded by the *trp* RBS and the distance from *k*-gene termination codon to the RBS of *Fd* gene is 45 bp. Plasmids were prepared and propagated in the *E. coli* strain HB101 (*recA*1, *endA*1).

EXPERIMENTAL AND DISCUSSION

(a) Expression of soluble Fab-fragments in the *Escherichia coli* cytoplasm

In an earlier study (Cabilly et al., 1984), we constructed expression vectors for κ-chains and Fd fragments. The signal peptide coding sequence of each of the Ig genes was replaced by an ATG start codon and, as a result, Ig products accumulated in the *E. coli* cytoplasm. To obtain co-expression of both κ-chain and Fd fragments, *E. coli* cells were co-transformed by two expression plasmids each carrying a different selection marker. However, no control could be established over the relative amount of each plasmid within individual cells. For this reason, and due to the possibility that translation of the two polypeptides from the same mRNA might improve their chance for proper assembly, a single plasmid harboring both genes in one transcription unit was constructed. Details of the plasmid, marked pFabCEA, are presented in Fig. 1.

Transformants carrying pFabCEA expressed both κ-chains and Fd fragments (Fig. 2A; note that the relative intensities of the two bands might be affected by the differential sensitivity of the Ig polypeptides to detection by rabbit anti-mouse IgG; Cabilly et al., 1984). The amounts of soluble Ig polypeptides in *E. coli* transformants growing at three different temperatures (37, 30 and 21°C) were compared. Relative band density of immunoblots (Fig. 3, lanes 4–6) show that the amount of soluble Ig polypeptides is about ten-fold higher in cells growing at 21 or 30°C as compared to cells grown at 37°C. In addition, at the lower temperatures, a significantly higher amount of insoluble Igs was also observed (Fig. 3).

Ig polypeptides from the soluble fraction of cultures grown at 21°C were purified on a DE-52 column and analysed by non-reducing SDS-PAGE. Several bands were detected when the blotted membrane was reacted with rabbit anti-mouse IgG (Fig. 2B). The broad band at about 47 kDa represents two nearly overlapping bands (lanes 1, 2). These bands correspond in size to Ig dimers. The predicted M_r of a κ/Fd dimer is 47 508 and that of a κ-chain dimer is 46 483. It therefore seems that the lower of the two high M_r bands is a κ-chain dimer, whereas the higher one is a κ/Fd dimer. The Ig preparation contains additional lower M_r bands. These bands represent either monomers or non-covalent dimers which dissociate in the presence of SDS. In bacteria producing κ-chain only, a band which probably represents κ-chain dimers was detected (lane 3).

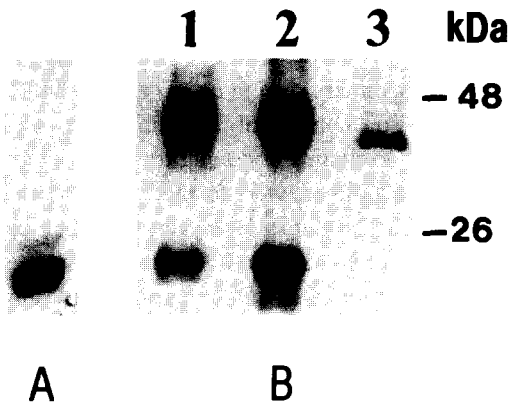

Fig. 2. Immunoblot of B-Fab under reducing and non-reducing conditions. Panel **A**: analysis of Igs from B-Fab-producing cells under reducing conditions. Panel **B**: non reducing gel. Lane 1, Ig-polypeptides from Fab-producing bacteria; lane 2, a preparation (the same as in lane 1) that has been further purified by affinity chromatography on anti-mouse IgG-agarose; lane 3, a preparation from κ-chain-producing bacteria. Transformants of the *E. coli* strain W3110, AE2 *tonB-trpE*, were grown at 21°C in M9 medium supplemented with 4% L broth to a cell density of A_{550} = 1.0. The harvested cell pellet from a 1 liter culture was suspended in 5 ml of disruption buffer containing 20 mM Tris-HCl, pH 8, 20 mM EDTA, 1 mM PMSF and 1 mM leupeptin, disrupted by a French Pressure Cell, and centrifuged at 40 000 × *g* for 30 min. The supernatant was dialysed against 20 mM Tris · HCl, pH 8, 5 mM NaCl, and purified by two consecutive runs on a DE-52 column (5 ml, Whatman) pre-equilibrated and eluted with the same buffer. The first 10 ml of unbound material was collected, concentrated to 1.5 ml and dialysed against TS buffer. Samples representing 1 ml bacteria, A_{550} = 1.0 were separated on 0.1%SDS–12% PAGE, and the proteins transferred to nitrocellulose membranes (Schleicher & Schuell, BA 83). The membranes were blocked with 1% BSA in TS buffer, incubated with affinity purified rabbit anti mouse IgG (1:1000) for 1 h at 37°C, washed three times for 10 min with TS buffer, incubated with 1:1000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate for 1 h, and washed again with TS buffer. The bands were visualized by the method of Leary et al. (1983).

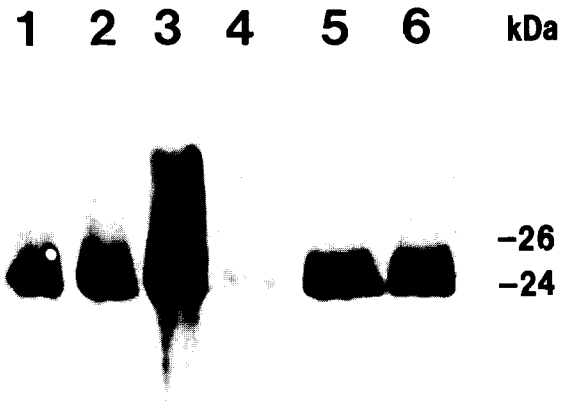

Fig. 3. Expression of Ig polypeptide in *E. coli* as a function of the growth temperature. Bacteria (10 ml) were grown to a cell density of A_{550} = 1.5. The cell pellet was suspended in 1 ml disruption buffer (see Fig. 2) containing 1 mg lysosyme and incubated for 30 min. at 4°C. Following freezing and thawing four times, cellular DNA was fragmented by shearing through a 27-gage syringe needle and the lysate centrifuged for 15 min in an Eppendorf centrifuge at 4°C. One tenth of either the particulate on the soluble fractions was applied to 0.1% SDS–12% PAGE. Immunoblots were developed by alkaline phosphatase Ab conjugate as described in Fig. 2. The particulate fractions (lanes 1–3) and the soluble fractions (lanes 4–6) were derived from cultures grown at 37°C (lanes 1,4), 30°C (lanes 2,5) and 21°C (lanes 3,6).

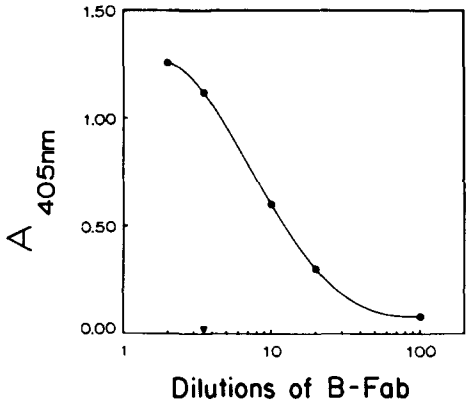

Fig. 4. A dose response curve of B-Fab binding to CEA. Analysis was done by EIA. (●), B-Fab; ▼, purified extracts from bacteria producing either κ-chain or Fd fragments (see Fig. 2). The amount of Ig chains in the different 'non-diluted' DE-52 purified extracts was brought to about 3 μg/well.

CEA (25 ng in 50 μl of 0.1 M carbonate-bicarbonate buffer, pH 9.3) was dried at 37°C in wells of a polyvinylchloride microtiter plate and nonspecific sites were blocked with 2% BSA. Serial dilutions of Ig purified from *E. coli* extracts were added and incubated for 90 min at 37°C, then washed extensively with phosphate-buffered saline containing 0.05% Nonidet-P40 (PBS-NP40). Goat anti-mouse Fab-alkaline phosphatase conjugate was used to determine specific ligand binding. Parallel series of diluted Ig preparations were incubated in the absence of antigen for background subtraction.

(b) Ag binding by bacterially-produced Fab

DE-52-purified Igs from *E. coli* extracts were analysed for Ag (CEA) binding by EIA. As shown in Fig. 4, B-Fab binds to CEA in a saturable manner. In contrast to B-Fab, no Ag binding was detected when either κ-chains or Fd-fragments, were analysed under the same conditions (Fig. 4). The specificity of B-Fab is also demonstrated by its ability to compete for the binding of hybridoma-produced ^{125}I-anti-CEA to the antigen (Fig. 5). This demonstrates that

the bacterially-produced Fab has the same antigen binding site as that of the monoclonal antibody from which it is was derived.

On the basis of these results, the estimated amount of functional B-Fab obtained after DE-52 purification from 1 ml of bacteria at A_{550} = 1.0, is about 100 ng. This amount is equivalent to 5–10% of the total protein in the DE-52 eluate or about 1.5 μg/mg protein.

The activity of the DE-52-purified B-Fab remained stable upon storage at 4°C for more than two months.

(c) Conclusions

(1) This report shows that functional Fab fragments can be obtained from extracts of *E. coli* transformants.

(2) The amount of Ig polypeptides and its soluble fraction is much higher in *E. coli* cells growing at 21°C or 30°C rather than at 37°C.

(3) The soluble Fab fragments isolated from *E. coli* appear as covalent dimers (Fig. 2B). It seems that in the highly reducing environment of the *E. coli* cytoplasm (Politt and Zalkin, 1983), the Ig polypeptides exist as non-covalently linked dimers, and that the covalent dimers are formed by air oxidation following cell rupture.

(4) The Ig dimers seen in *E. coli* are not limited to pairs of κ/Fd. Dimers of κ-chains were seen in extracts of κ-chain producing bacteria (Fig. 2B, lane 3) and Fd dimers were formed in Fd producing cells (unpublished data). However, the much higher association constant of κ/Fd dimers compared to that of the homodimers (Dorrington, 1978), implies predominance of κ/Fd dimers in cells producing equal amounts of both chains.

(5) Since in the B-Fab expression plasmid the κ-chain coding sequence is located upstream from the Fd fragments, an excess of κ-chains may be expected. This would explain the apparent production of κ-chain dimers in Fab-producing cells (Fig. 2B).

(6) This method opens a route for in situ screening of *E. coli* colonies producing Fab fragments with particular specificities.

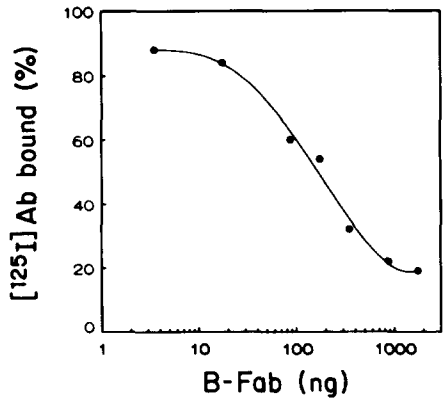

Fig. 5. Inhibition curve of ^{125}I-labeled anti-CEA binding to CEA with B-Fab as inhibitor. Increasing amounts of DE-52 purified B-Fab were added together with 50 ng of ^{125}I-anti-CEA (Johnson and Thorpe, 1982) to CEA precoated wells. Following incubation for 90 min, the wells were washed with PBS-NP40, cut out and taken for radioactive measurement in a gamma counter. The total amount of Ig polypeptides in the DE-52 purified extracts (indicated as B-Fab on the abscissa) was estimated by scanning immunoblots which included standards of hybridoma-produced anti-CEA and were visualized by goat anti-mouse Fab-alkaline phosphatase conjugate.

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