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Periplasmic Expression of a Recombinant Antibody (MabB9) in *Escherichia coli*

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Expression in the periplasm of *Escherichia coli* of cloned heavy and light chain cDNAs for Fab fragment of a murine monoclonal antibody MabB9 (γ2b, κ), specific for human plasma apolipoprotein B-100 of LDL, was studied. For the purpose, a vector for two-cistronic expression of the heavy chain cDNA, at the 5' terminus, and light chain cDNA, at the 3' terminus, was constructed using the signal sequences, *pelB* (for heavy chain) and *ompA* (for light chain) in a pET vector system. The constructed vector was transformed into *E. coli* BL21(DE3). The expressed heavy chain (25 kDa) and light chain (23 kDa) of the antibody molecule were detected in total cell extracts as well as in the periplasmic extracts of *E. coli*.

Monoclonal antibody technology has provided cell culture methods for isolation and propagation of antibody molecules (6).

The manipulation of immunoglobulin genes by recombinant DNA technology significantly extends the utility of monoclonal antibodies and allows the construction of antibody derivatives (3, 10).

Escherichia coli offers efficient transformation and rapid inexpensive fermentation. The ease of genetic manipulation in *E. coli* allows cloning and mutagenesis, directional or random, for the production of designed antibody molecules. This antibody engineering provides many fragment antibodies which are valuable immunoreagents for the construction of diagnostic kits and are also useful for studying the structure-function relationship. The efficient expression of antibody fragments in bacteria is of great industrial importance. Many investigators have tried to clone and express many types of antibody fragments in *E. coli* (1, 12, 14-16).

Apolipoprotein B-100 is the major protein moiety (95-100%) of low-density lipoproteins (LDL) in plasma. LDL and LDL-cholesterol in human plasma are clinically evaluated as important markers, whose concentrations have positive correlations with the incidence of coronary artery diseases. We have previously reported about the cloning and sequence characterization of cDNAs coding for heavy and light chains of a monoclonal antibody, MabB9 (γ2b, κ), specific for human plasma apolipoprotein B-100 (7).

In this paper, we report construction of a bacterial expression system for MabB9 recombinant antibody, which allows production of antibody fragments directly out of periplasmic extracts. For the purpose, a vector for two-cistronic expression of the heavy chain cDNA, at the 5' terminus, and light chain cDNA, at the 3' terminus, was constructed using *pelB* (for heavy chain) and *ompA* (for light chain) signal sequences in a pET vector system.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Medium

The bacterial strains and plasmids used in this experiment are listed in Table 1. *E. coli* strains NovaBlue, BL21(DE3), and plasmid vectors pT7Blue and pET29b(+) were purchased from Novagen (Madison, WI, U.S.A.). *E. coli* NovaBlue was used as a recipient for routine transformation. *E. coli* BL21(DE3) was used as an expression host for the genetically engineered antibodies. Transformants were grown in LB medium (13) containing Ap (50 μg/ml) or Km (30 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 40 μg/ml), and 0.2 mM isopropyl thiogalactoside (IPTG).

Polymerase Chain Reaction (PCR)

To construct a two-cistronic expression vector for the *E. coli* periplasmic secretion of heavy and light chains of the Fab fragment of the MabB9 antibody, PCR was performed, and nucleotide sequences coding for *pelB* and *ompA* signal sequences were introduced to the 5' of B9 heavy and light chain cDNA sequences, respectively.

PCR was performed under the following conditions: 2

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Table 1. The bacterial strains, and plasmids used in this experiment.

Strains or plasmid	Description	Reference
strains		
E. coli NovaBlue	*endA1 hsdR17* (r_K^-, m_K^+) *supE44 thi recA1 gyrA96 relA1 lac*[F'*proAB lacI*^{q}ZΔM 15 ::Tn*10*(tet^r)] (DE3)	Novagene
E. coli BL21(DE3)	*hsdS gal*(λ*elts 857 ind1 sam7 nin5 lacUV5-T7 gene1*)	18
plasmid		
pB9HT7	pT7+MabB9 Heavy chain DNA fragment (cloning site: *Eco*RV)	7
pB9LBlue	pBluescript+MabB9 Light chain DNA fragment (cloning site: *Sma*I)	7
pET-29b(+)	K_m^r T7*lac lacI* 5371bp	18
pT7Blue	Ap^r *lacZ* 2887bp	9
pETB9L	pET29b(+)+Recombinant MabB9 light chain by PCR	this work
pETB9H	pET29b(+)+Recombinant MabB9 heavy chain by PCR	this work
pETB9HL	pET29b(+)+Recombinant MabB9 heavy & light chain by PCR	this work

pmoles of primers and 5 units of amplified DNA polymerase (Perkin-Elmer, Foster, CA, U.S.A.) were added to the template DNA. 30 cycles of pre-denaturation at 90°C for 60 sec, denaturation at 95°C for 10 sec, annealing at 52°C for 10 sec and elongation at 72°C for 20 sec were performed. Post-elongation at 72°C for 120 sec was performed as a finishing step.

DNA Sequencing

All the sequences introduced by PCR were confirmed by DNA sequencing by the dideoxy chain-termination method (14), using an M13 universal primer or specific internal synthesized primers, and a Sequenase™ version 2.0 kit (United States Biochemical, Cleveland, OH, U.S.A.).

DNA Manipulation

All the standard manipulations of DNA, and plasmid transformations of *E. coli* were performed as described by Maniatis *et al.* (13).

Restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, WI, U.S.A.), Takara (Tokyo, Japan) and Boehringer Manheim (Indianapolis, IN, U.S.A.). [α-^{35}S]dATP was purchased from Amersham (Arlington Height, IL, U.S.A.).

Elution of DNA fragments from agarose gels was carried out using Gene Clean kit (Bio101 Inc, Lajolla, CA, U.S.A.). Oligonucleotides were synthesized using an Applied Biosystems (Foster, CA, U.S.A.) model 350A DNA synthesizer at Bioneer (Cheongwon, Korea).

Expression of Fab Fragment of MabB9 in *E. coli*

Single colonies of BL21(DE3) freshly transformed with the expression vector were inoculated in LB medium containing 30 μg/ml of kanamycin. One % of overnight cultures were transferred to 50 ml of LB containing 30 μg/ml of kanamycin in a 250 ml Erlenmeyer flask. The cells were incubated at 37°C with vigorous shaking until OD_{600} reached 0.4~1.0. Then, 1 mM of IPTG (final conc.) was added for induction and the cells were maintained with shaking for an additional 2~3 h. For harvesting, the flask was quickly chilled on ice for 5 min and the cells were harvested by centrifugation at 5,000× g for 5 min at 4°C. The cells were washed once in 1/4 culture volume of a cold buffer (50 mM Tris-HCl, pH 8.0 at 25°C, and 2 mM EDTA) and stored as frozen pellets at -70°C for future use.

Preparation of Periplasmic Extract

1.5 ml culture of cells was centrifuged in a microfuge for 1 min. Supernatant was decanted briefly and the cells were resuspended in the residual volume of the culture medium. 15 μl of chloroform was added, and the tubes were vortexed and left at room temperature for 15 min. 75 μl of 10 mM Tris-HCl (pH 8.0) was added and the samples were centrifuged at 12,000×g for 15 min. The supernatant was transferred to a fresh tube and an equal volume of 2× SDS sample buffer (8) was added. The samples were heated at 70°C for 3 min and loaded onto SDS-polyacrylamide gel for protein analysis.

Preparation of Total Cell Extract

E. coli cells were collected by centrifugation and washed in 1/10 culture volume of a buffer (10 mM Tris-HCl, pH 8.0). After centrifugation, the cells were resuspended in 1/10 culture volume of SDS sample buffer by vortexing. The samples were heated at 70°C for 5 min and proteins were analyzed by SDS-PAGE.

SDS-PAGE and Western Blotting

Proteins were separated by 15% SDS-PAGE (8) for 3~4 h at 30 mA and transferred to 0.45 m nitrocellulose membrane (Schleicher & Schule, NH, U.S.A.) electrophoretically for 3~4 h at 170 mA, as described by Towbin *et al* (19). Following the transfer, the membrane was submerged in TBS buffer (20 mM Tris-HCl, 0.5% HCl, pH 7.5) containing 3% gelatin for 2 h at 37°C with gentle shaking, to block nonspecific binding. The membrane was washed three times in TBS, and incubated in 100 ml of TBS containing 1% gelatin and 30 μl of horseradish peroxidase-conjugated, goat anti-mouse IgG(H+L) (BioRad, Hercules, CA, U.S.A.) for 3 h. The membrane was washed three times for 5 min each and soaked

in the substrate solution (cold methanol 20 ml, HRP developer 60 mg, H_2O_2 100 μl, TBS 100 ml). Positive bands were observed within 3~5 min. The substrate solution was then discarded and the membrane was washed with several changes of deionized water.

RESULTS

Construction of Secretional Expression Vectors for Heavy and Light Chains of MabB9

To construct expression vectors for periplasmic secretion of the heavy and light chains of the Fab fragment of MabB9, the heavy chain cDNA and light chain cDNA with *pelB* and *ompA* signal sequences at their 5' termini, respectively, were prepared by PCR from previously cloned templates (7). Detailed experimental procedures for the PCR are represented schematically in Fig. 1. The primer sequences used are listed in Table 2. These PCR products were first ligated into pT7Blue vector for color selection of recombinant clones. Plasmids were prepared from positive clones and used for DNA sequencing experiments. All the sequences introduced by PCR were thus checked for any errors in Taq DNA polymerase. The confirmed DNA constructs of the heavy and light chain DNAs were ligated into the pET29b(+) vector, and analyzed for the expression of recombinant antibodies.

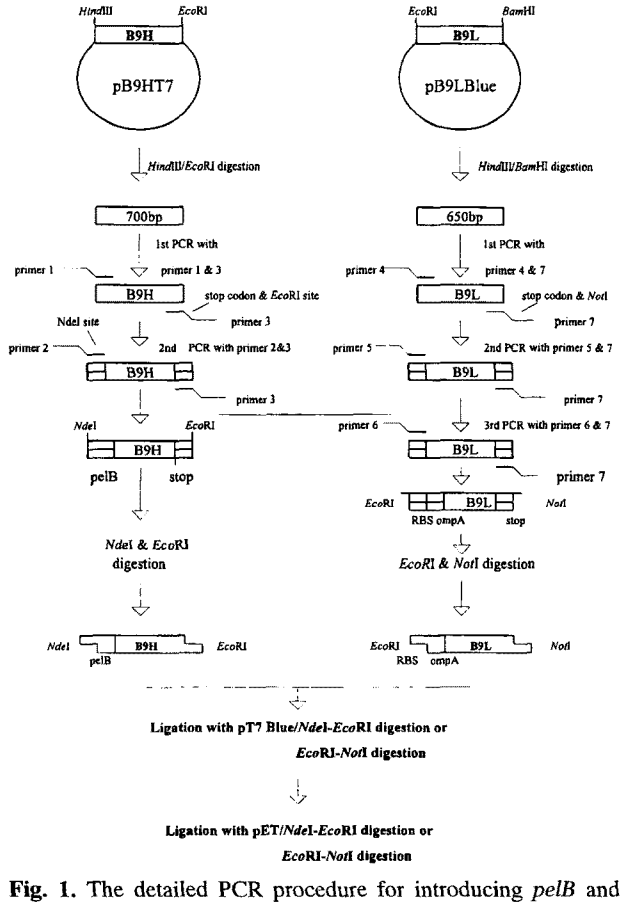

Fig. 1. The detailed PCR procedure for introducing *pelB* and *ompA* signal sequences to the 5' of heavy and light chain cDNAs for Fab fragment MabB9 antibody.
pelB sequence was introduced to the B9 heavy chain cDNA (230 amino acids [aa]). Ribosomal binding site (RBS) and *ompA* sequences were intorduced to the B9 light chain cDNA (214 aa).

Expression, Localization and Identification of Recombinant Antibodies of MabB9

Fig. 2 shows the expression plasmids and their detailed construction. These plasmids were designed for periplasmic expression of only the heavy chain or only the light chain, or for the expression of both chains of the MabB9 Fab fragment. All the plasmids contain signal sequences to direct secretion to the periplasm. pETB9L is the expression plasmid for MabB9 light chain (V_L+C_L), and pETB9H is the expression plasmid for MabB9 heavy chain (V_H+C_H). pETB9HL is the plasmid for two-cistronic expression of both chains, the heavy chain and light chain.

For protein expression, the recombinant plasmid is transformed into an *E. coli* host strain, BL21(DE3), containing a chromosomal copy of the gene for T7 RNA polymerase. The host cell is a lysogen of bacteriophage DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA plymerase (18). Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase is the *lacUV5* promoter, which is inducible by IPTG. Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

The pET expression plasmid contains a T7*lac* promoter system, where a *lac* operator sequence is located just downstream of the T7 promoter (17). It also carries the natural promoter and coding sequences of the *lac* repressor (*lacI*), which are oriented so that the T7*lac* and *lacI* promoters diverge. When this type of vector is used in DE3 lysogens, the lac repressor acts not only at the *lacUV5* promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase, but also at the T7*lac* promoter in the vector to block transcription of the target gene by any T7 RNA polymerase that is made.

Fig. 3 shows the result of SDS-PAGE and Western blot analysis of total cell extracts harboring the expression plasmids. The expressed heavy chain (25 kDa) or light chain (23 kDa) band was clearly observed by Coomassie blue staining after SDS-PAGE in all the cases of expression plasmids used (Fig. 3A). But, in Western blot analysis, the expressed heavy chain didn't exhibit a good reaction with the HRP-coupled 2ndary an-

Table 2. Primer sequences in this experiment.

Primer No.	Sequences	Length (bp)
1	5'-CTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCGAGGTGCAGCTGGTGGAGTCTGGAGCT-3' L L L L A A Q P A M A — B9H N-terminal	60
2	5'-GTATCATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCC-3' *Nde*I M K L L P T A A A G L Y L L L A A	58
3	3'-TCGCCCGGGTAAAGTTGTTAGTTGGGGACAGGAGGTACGACTCTTAAGGCGG-5' B9H C-terminal — Stop *Eco*RI	52
4	5'-GGTTTCGCTACCGTAGCGCAGGCCGATATCAAGATGACCCAGTCTCCATCT-3' G F A T V A Q A — B9L N-terminal	51
5	5'-ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCG-3' M K K T A I A V A L A G F A T V A	57
6	5'-GCATGAATTG CTGCACAAGGAGAAAAATAAAATGAAAAAGACAGCTATC-3' *Eco*RI RBS M K K T A I	49
7	3'-AGTTGTCCTTACTCACAACT CGCCGGCGTGAG-5' B9L C-terminal — Stop *Not*I	32

pelB signal=5'-MKYLLPTAAAGLLLLAAQPAMA-3'. *ompA* signal=5-'MKKTAIAIAVALAGFATVAQA-3'.

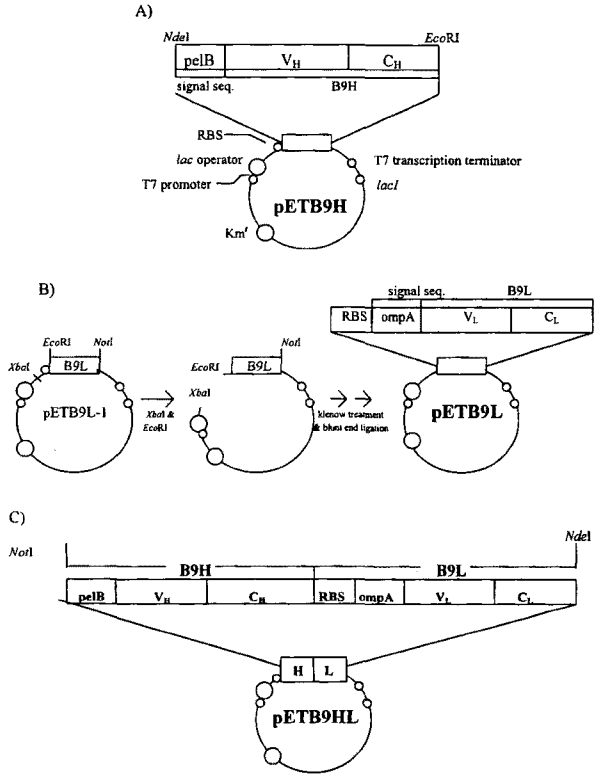

Fig. 2. Construction of secretional expression plasmids for heavy and light chains of MabB9 recombinant antibody.
(A) *Nde*I-*Eco*RI fragment of heavy chain cDNA including *pelB* signal sequence was ligated into pET29b(+) to produce pETB9H. (B) *Xba*I-*Eco*RI cut of pETB9L-1 was combined with *Eco*RI-*Not*I fragment of light chain cDNA including RBS and *omp* A signal sequences to produce pETB9L. (C) pETB9HL has two-cistronic expression system for the heavy at 5' terminus and light chain at 3' terminus.

tibodies (Fig. 3B, lane 2). When the incubation steps for binding with 2ndary antibodies and color reaction were prolonged, a weak band of corresponding size (25 kDa) appeared (data not shown). We checked the reaction of 2ndary antibodies with purified Fab fragment of native monoclonal antibody, MabB9. And here also, the reaction with heavy chain was very poor. Therefore, it is concluded that biased type-specificity of the commercially obtained 2ndary antibodies has produced a poor reaction with our *r*2b type of MabB9 heavy chain. The two bands seen in the Western blotting (lane 4 of Fig. 3B) are believed to be generated by uncomplete processing of the signal peptide attached (about 20 amino acids). As a control, no band of predicted size was observed in total extracts of cells harboring the pET29b(+) control vector.

Fig. 4 shows the result of SDS-PAGE and Western blot analysis of periplasmic extracts of cells harboring the expression plasmids. Distinct bands were not observed in the Coomassie blue staining (Fig. 4A), but positive bands of the heavy chain (25 kDa) and the light chain (23 kDa) of the Fab fragment of MabB9 were observed by Western analysis (Fig. 4B). This means that the expressed heavy and light chains of recombinant antibody have been properly secreted into the periplasm of *E. coli*, using the signal sequences that we have introduced. But again, the heavy chain from pETB9H could hardly be detected due to the poor reaction of the 2ndary antibodies, while the periplasmic expression proteins from cells transformed with pETB9L and pETB 9HL could be well identified. As a result preparation or use of other commercial sources for the 2ndary antibodies is necessary on this regard.

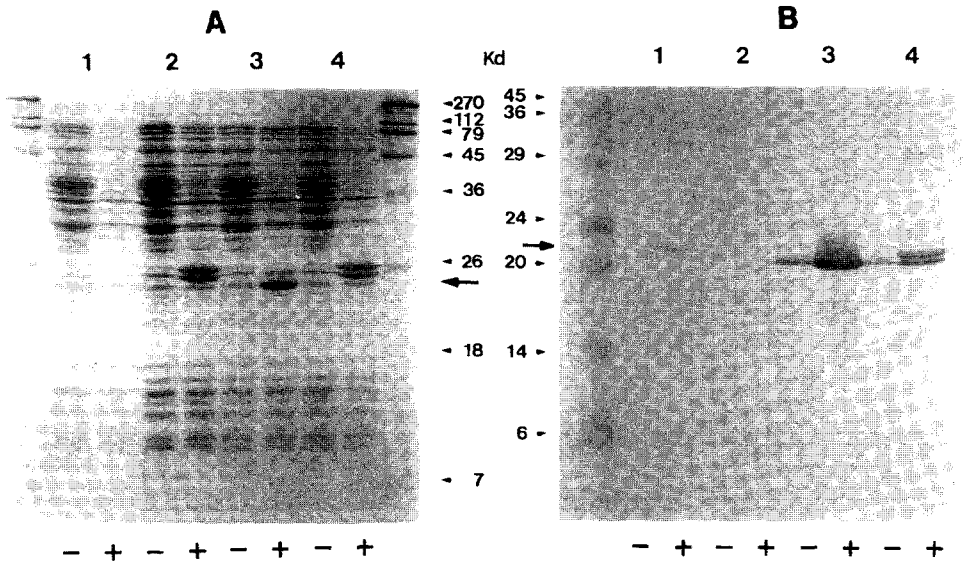

Fig. 3. SDS-PAGE and Western blot analysis of total cell extracts of cells harboring the expression plasmids of MabB9 recombinant antibody.

Samples were separated by 15% SDS-PAGE and stained with Coomassie blue (panel A). Detailed conditions of Western blot analysis (panel B) are described in MATERIALS AND METHODS. Lane 1, *E. coli* BL21(DE3) harboring pE29b(+) control vector; 2, *E. coli* BL21(DE3) harboring pETB9H; 3, *E. coli* BL21(DE3) harboring pETB9L; 4, *E. coli* BL21(DE3) harboring pETB9HL. +, 1 mM IPTG induction; -, no induction.

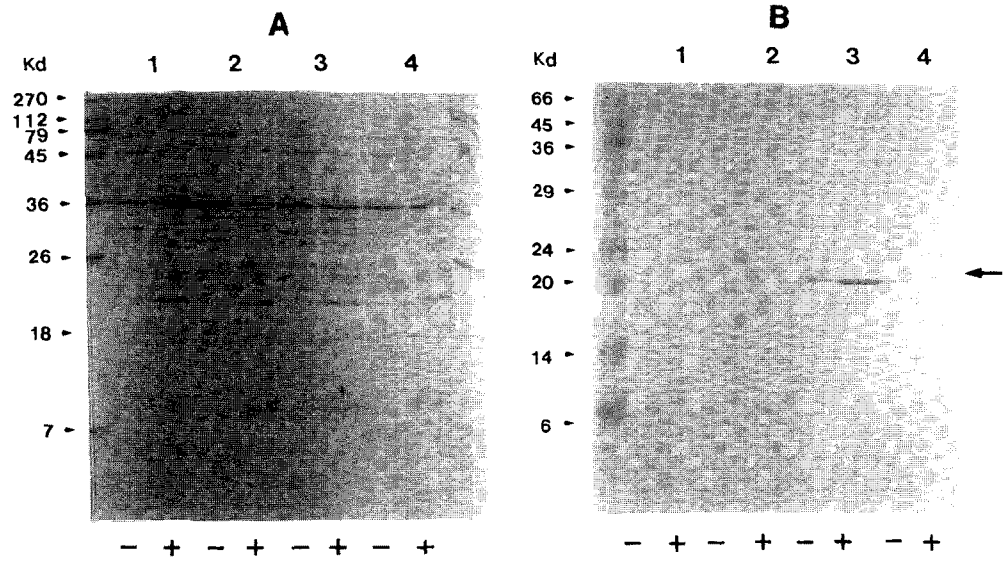

Fig. 4. SDS-PAGE and Western blot analysis of periplasmic extracts of cells harboring the expression plasmids of MabB9 recombinant antibody.

Samples were separated by 15% SDS-PAGE and stained with Coomassie blue (panel A). Detailed conditions of Western blot analysis (panel B) are described in MATERIALS AND METHODS. Lane 1, *E. coli* BL21(DE3) harboring pE29b(+) control vector; 2, *E. coli* BL21(DE3) harboring pETB9H; 3, *E. coli* BL21(DE3) harboring pETB9L; 4, *E. coli* BL21(DE3) harboring pETB9HL. +, 1 mM IPTG induction; -, no induction.

DISCUSSION

Whole antibody molecules can be expressed in considerable amounts in eukaryotic (11) and yeast cells (20), but poorly in *E. coli* cells (2, 5). On the other hand, parts of the antibody (Fv, Fd, Fab) that are small, but still have antigen-binding ability can be expressed efficiently in *E. coli* (1, 4, 15, 16).

Here we reported on the bacterial expression of genetically engineered antibodies. A series of plasmids has

been designed so that the heavy and light chains of the Fab fragment of a murine monoclonal antibody could be synthesized and secreted into the periplasmic space in *E. coli*.

Periplasmic expression is advantageous in many ways. First, the signal peptide is cleaved off in the process of secretion, leaving the mature protein without an N-terminal methionine. Second, periplasmic space provides a more oxidative environment than the cytoplasm, thereby favoring the S-S bond formation which is necessary for forming an antibody structure. Third, there are fewer proteases and other proteins in the periplasm, facilitating the purification of antibody molecules from other contaminants. By contrast to expression in the cytoplasm, it has been reported that a strong promoter or culturing of the host cell at 37°C is not always effective for expression, probably because the secretion is coupled with translation of the protein (12). Secretion may be the limiting factor for high level production in the periplasm. According to our results, the target proteins were synthesized well in *E. coli*, but the majority of them were located in the cytoplasm not in the periplasm. To improve secretion, further optimization studies of the promoter system and culture conditions are necessary.

This study is valuable as it demonstrates the possibility of high level expression of a recombinant antibody (MabB9) protein in *E. coli*: The portion of the recombinant antibody expression achieved in total cell proteins was as high as 25~30%.

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