Volume 244, number 2, 383–387 FEB 06848 February 1989

# Expression of ricin B chain in *Escherichia coli*

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Received 23 December 1988

DNA encoding ricin B chain was fused to that encoding the *E. coli* OmpA signal peptide using the expression secretion vector pIN-III-ompA. When induced, *E. coli* cells transformed with the recombinant plasmid express ricin B chain. The recombinant product accumulates in the periplasmic space in a soluble, biologically active form.

Ricin B chain; Expression; Lectin; Immunotoxin; (*E. coli*)

## 1. INTRODUCTION

Ricin, the cytotoxic lectin from *Ricinus communis* seeds, is a heterodimer consisting of a toxic polypeptide (the A chain) joined to a cell-binding, galactose-specific lectin (the B chain) by a single disulphide bond (review [1]). The A chain catalytically inactivates eukaryotic ribosomes by specifically depurinating 28 S ribosomal RNA [2,3]. Penetration of the A chain into the cytoplasm of target cells begins when the holotoxin binds to cell surface galactose residues and is achieved when the B chain facilitates the translocation of A chain across the membrane of an intracellular compartment during subsequent endocytosis [4,5]. The two B chain functions (cell binding and A chain translocation) are believed to reside on separate domains of the polypeptide [6], although this remains to be convincingly established [7].

Whole ricin or its purified A chain have been widely used for the construction of immunotoxins (review [8,9]). Immunotoxins are hybrid molecules in which cytotoxins or their toxic subunits are chemically coupled to cell-reactive monoclonal antibodies. At present the in vivo application of whole ricin immunotoxins is not feasible since the ricin B chain galactose-binding capacity overrides the cell-type targeting specificity conferred by the antibody [9].

One approach to this problem is to genetically modify ricin B chain using protein engineering techniques. The ricin precursor gene has been cloned [10,11] and B chain residues responsible for galactose binding have been identified [12]. Such residues could be modified by oligonucleotide site-specific mutagenesis and expression of DNA encoding modified ricin B chain. It is hoped that this approach might produce mutant B chain no longer able to bind galactose but still capable of potentiating A chain translocation. A prerequisite for this work is the ability to express biologically active, recombinant ricin B chain. Previous work has shown that this can be achieved in eukaryotic hosts if the recombinant B chain is directed into the endomembrane system using homologous or heterologous eukaryotic signal sequences [13–15]. In the present paper we show that biologically active ricin B chain can also be produced in *E. coli* by using a bacterial signal sequence which targets the product to the periplasmic space.

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*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/89/$3.50 

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

*Escherichia coli* strains TG2 (*Δ(lacpro) thi, staA, hsdR⁻, hsdM⁻, recA⁻*, sclC 300::Tn10 F′, *traD36, proAB, lacI^q, lacZ* DM15), DH1 (F⁻ *recA1, evdA1, gyrA96, thi-1, hsdR17* ($r_k^- m_k^+$, *Supt44, relA1*)) and JA221 (*lpp⁻, hsdM⁺, trpt5, leuB6, lacY, recA1*, F′*lacI^q*, *lac⁺*, pro⁺) were used as indicated. Restriction endonucleases, $T_4$ ligase and biotinylated protein A/streptavidin-horseradish peroxidase were obtained from Amersham (Bucks, England) and isopropyl β-D-thio-galactopyranoside (IPTG), ampicillin and asialofetuin were obtained from Sigma (Poole, England). Vector pIN-111-ompA3 [16] and *E. coli* strain JA221 were kindly provided by Dr M. Inouye.

### 2.2. *General methods*

Plasmid DNAs were prepared by the alkaline lysis method [17] and purified by centrifugation in cesium chloride-ethidium bromide. Restriction enzyme digests and ligations were carried out as recommended by the suppliers.

### 2.3. *Construction of pIN-111-ompA3 ricin (R)B*

To avoid possible expression of a truncated ricin B chain caused by translation initiation at Met76, utilising the upstream sequence GGAG as a Shine-Dalgarno sequence (unpublished), Met76 was converted to an Ile codon by oligonucleotide site-directed mutagenesis using standard procedures [18] (Grierson, C., unpublished). This was accomplished in M13 using a ricin B chain encoding *Bam*HI fragment as a template and the DNA was recloned into pUC19 to generate pTCAO.3. The 5′-end of this ricin B chain encoding sequence was then excised with *Bam*HI and *Kpn*I and the 328 bp fragment ligated with the 900 bp *Kpn*I-*Sal*I fragment of pUC8pRB (a plasmid containing ricin B chain preceded by the preproricin signal sequence) and with the large *Bam*HI-*Sal*I fragment of expression vector pIN-111-ompA3 in a tripartite ligation. This strategy is summarised in fig.1.

### 2.4. *Expression of ricin B chain in* E. coli

Recombinant plasmid pIN-111-ompA3RB was introduced into *E. coli* by calcium chloride-mediated transformation. Bacteria were grown at various temperatures (see section 3) in the presence of ampicillin (50 μg/ml) and IPTG (1 mM) in either (a) nutrient broth supplemented with glucose (0.4%), $MgSO_4$ (10 mM) and $MgCl_2$ (10 mM), or (b) defined medium containing casamino acids (1%), glucose (0.4%), $MgSO_4$ (10 mM), $MgCl_2$ (10 mM), tryptophan (40 mM), $CaCl_2$ (100 μM), vitamin B1 (1 mM), $Na_2HPO_4$ (4 mM), $KH_2PO_4$ (2 mM), NaCl (8 mM) and $NH_4Cl$ (20 mM). Cultures were grown to an $A_{550}$ of 0.6–0.8 and the cells were harvested by centrifugation. To prepare the periplasmic fraction, the cells (1 l culture) were resuspended in 20 ml of 10 mM Tris-HCl, pH 7.5, containing 20% (w/v) sucrose and kept on ice for 10 min. After repelleting the cells by centrifugation at 4°C, they were resuspended in 10 ml of 10 mM Tris-HCl, pH 7.5, supplemented where appropriate with 1 mM lactose, and kept on ice for a further 10 min. After centrifugation at 18000 rpm for 20 min, the supernatant was taken as the periplasmic fraction. Where appropriate, this fraction was further centrifuged at 100000 × *g* in a Beckman airfuge.

### 2.5. *Identification of recombinant B chain*

Samples, including crude lysates and periplasmic fractions, were analysed by SDS-polyacrylamide gel electrophoresis [19] and Western blotting [20]. Blots were probed with polyclonal antibodies raised in rabbits against ricin B chain purified from *Ricinus communis* seeds [21], and were developed using the biotinylated protein A/streptavidin-peroxidase system (according to the suppliers' instructions). Ricin B chain was enzymically deglycosylated using endo-*N*-acetylglucosaminidase H [22].

### 2.6. *Lectin activity of recombinant ricin B chain*

Recombinant ricin B chain present in various fractions was assayed for lectin activity by its ability to bind to immobilised asialofetuin, a glycoprotein with terminal galactose residues. The assay was a modification [14] of a procedure described previously [23]. Briefly, asialofetuin was used to coat the wells of a microtitre plate, samples containing recombinant B chain were added and, after washing, biologically active ricin B chain bound to the asialofetuin was measured by adding rabbit anti-ricin B chain antibodies followed by $^{125}$I-labelled protein A. By comparison to a calibration curve produced using native ricin B chain, this radioimmunoassay was used to quantify recombinant ricin B chain production.

## 3. RESULTS AND DISCUSSION

Previous studies have shown that biologically active recombinant ricin B chain can be expressed in eukaryotic cells when the recombinant product is directed into the endomembrane system of the host cell using an appropriate N-terminal signal sequence [13–15]. Cytoplasmically expressed recombinant ricin B chain aggregates, presumably because of incorrect folding and aberrant disulphide bond formation, and is devoid of biological activity (unpublished). In *E. coli*, the expression of eukaryotic proteins stabilised by intramolecular disulphide bonds may lead to the intracellular accumulation of inactive polypeptide aggregates [24]. When the expressed product is directed into the *E. coli* periplasmic space, however, the recombinant protein often folds into the biologically active, native conformation and forms the correct disulphide bonds.

Inouye and his colleagues [16] have developed a high expression secretion vector, pIN-111-ompA, which targets recombinant products to the periplasmic space of *E. coli*. This vector contains the signal sequence of a major *E. coli* outer membrane protein, the OmpA protein, to which the heterologous protein is fused. Secretion into the periplasmic space is accompanied by OmpA signal peptide cleavage and disulphide bond formation,

resulting in a correctly folded, biologically active recombinant protein [25–29].

We have followed this strategy to produce recombinant ricin B chain in *E. coli*. *E. coli* TG2 cells were transformed with vector alone or with a recombinant plasmid containing the ricin B chain DNA insert (pIN-111-ompA3RB, fig.1) and were grown at either 30 or 37°C. Total proteins present in various cell fractions after cell lysis were separated by SDS-PAGE and blotted onto nitrocellulose paper. A typical blot probed with anti-ricin B chain antibodies is shown in fig.2. Lysates from cells grown at 30°C (fig.2, lane 1) or 37°C (fig.2, lane 2) contained material which reacted strongly with the antibodies, and which had an apparent molecular mass equivalent to that of deglycosylated ricin B chain (fig.2, lane 8). This product was not detected in lysates from cells transformed with vector alone (fig.2, lane 3). Ricin B chain was detected in the periplasmic fraction prepared from cells grown at 30°C (fig.2, lane 4) and 37°C (fig.2, lane 5) and in the cell/debris pellet obtained after rupturing the outer membrane to release the periplasmic components (fig.2, lanes 6 and 7).

The bulk of the periplasmic ricin B chain produced by *E. coli* strain TG2 was soluble, although some aggregation had apparently occurred. Fig.3 shows that when the periplasmic fraction (fig.3, lane 2) was centrifuged at 100000 × *g* in a Beckman airfuge, ricin B chain was detected in both the supernatant (fig.3, lane 3) and in the pellet (fig.3, lane 4). (It should be noted that in the preparation of fig.3 the whole of the pellet was run in lane 4 but only 1/10 volume of the corresponding supernatant was run in lane 3.) Fig.3 also clearly indicates that the recombinant B chain product ran as a doublet. We assume that the smaller molecular mass band represents mature B chain from which the ompA signal peptide had been removed, while the less mobile band represents unprocessed product. The extent to

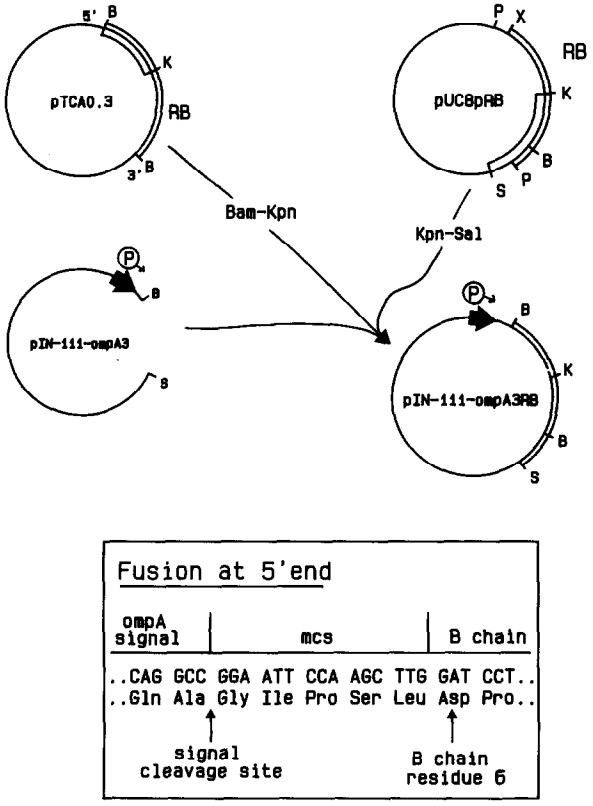


Fig.1. Construction of the ricin B chain expression plasmid. A 328 bp *Bam*HI-*Kpn*I fragment and a 900 bp *Kpn*I-*Sal*I fragment, which together contained the entire ricin B chain coding sequence, were ligated into *Bam*HI-*Sal*I cut pIN-111-ompA3 to produce pIN-111-ompA3RB. Upon expression of this construct the 5 N-terminal residues of native ricin B chain (Ala-Asp-Val-Cys-Met-) will be replaced with 5 residues from the multiple cloning site (MCS) (Gly-Ile-Pro-Ser-Leu-). Restriction sites indicated are B, *Bam*HI; K, *Kpn*I; S, *Sal*I; P, *Pst*I; X, *Xho*I.

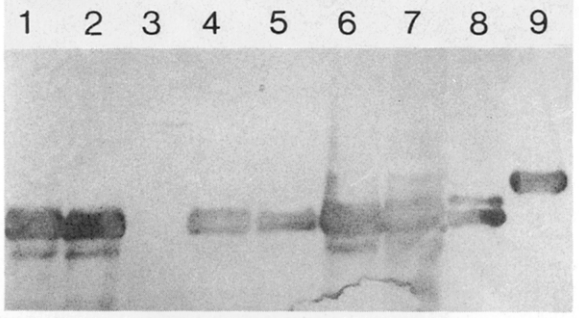


Fig.2. Expression of ricin B chain in *E. coli* TG2 cells. Transformants were grown at 30 or 37°C and, after lysis, proteins present in various fractions were separated by SDS-PAGE, blotted onto nitrocellulose and probed with anti-ricin B chain antibodies. Lanes: 1 and 2, total lysates from 30 and 37°C grown cells, respectively; 3, total lysate from cells transformed with vector lacking the ricin B chain DNA insert; 4 and 5, periplasmic fractions from 30 and 37°C grown cells; 6 and 7, cell pellet from 30 and 37°C grown cells; 8, deglycosylated ricin B chain (the upper band has had one oligosaccharide chain removed, the lower band has had both chains removed); 9, native ricin B chain.

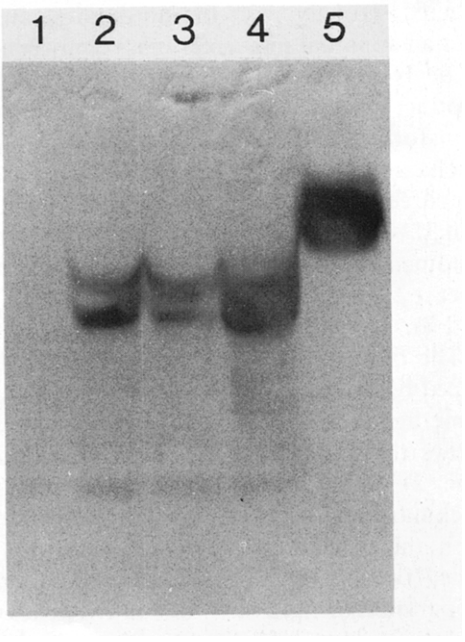


Fig.3. High-speed centrifugation of the periplasmic fraction from *E. coli* TG2 cells expressing ricin B chain. Proteins were separated by SDS-PAGE, blotted and probed with anti-ricin B chain antibodies. Lanes: 1, total lysate from cells transformed with vector lacking insert; 2, periplasmic fraction; 3, a 100 $\mu$l aliquot taken from the supernatant after high-speed centrifugation of 1 ml of periplasmic fraction; 4, the total pellet after high-speed centrifugation of 1 ml of periplasmic fraction; 5, ricin B chain.

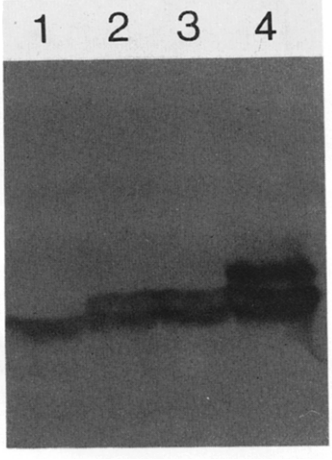


Fig.4. Expression of ricin B chain in different *E. coli* strains. *E. coli* strains JA221 (lane 1), TG2 (lane 2) and DHI (lane 3) were transformed with pIN-111-ompA3RB. Total lysates were probed for ricin B chain as for figs 2 and 3. Lane 4, deglycosylated ricin B chain.

Table 1

Effect of culturing conditions on the yield of biologically active ricin B chain expressed in *E. coli* strain JA221

| Media | Lactose (1 mM) | IPTG induction | Growth temp. (°C) | B chain ($\mu$g/l) | Control ($\mu$g/l) |
|---|---|---|---|---|---|
| NB | + | + | 30 | 1 | 0.1 |
| NB | + | + | 27 | 1.5 | 0 |
| NB | + | + | 25 | 12.5 | 0.3 |
| NB | − | + | 20 | 15 | 0.6 |
| NB | + | + | 20 | 20 | 0 |
| Defined | + | + | 20 | 100 | 0.5 |
| Defined | − | − | 18 | 24 | 0 |
| Defined | − | + | 18 | 800 | 0.7 |
| Defined | + | − | 18 | 400 | 0 |
| Defined | + | + | 18 | 1000 | 0.7 |

which the ompA signal sequence was processed was host strain dependent. Thus, while the recombinant product was expressed as a doublet in TG2 cells (fig.4, lane 2) or DHI cells (fig.4, lane 3), only a single processed species was produced by JA221 cells (fig.4, lane 1).

All three host strains produced biologically active B chain as determined by its ability to bind to immobilised asialofetuin. The amount of active B chain produced was markedly influenced by culturing conditions. Table 1 shows the effect of media, growth temperature, the presence of lactose in media and IPTG induction on recombinant B chain production by *E. coli* strain JA221. The optimal yield of recombinant product was observed for cells growing on defined medium supplemented with lactose at low temperature (18°C) after IPTG induction. For strain JA221, these conditions resulted in the production of 1 mg active ricin B chain per litre of culture. The actual level of expression was significantly higher than this but the radioimmunoassay used here only quantifies biologically active product. Upon storage this soluble, active product tended to aggregate with a concomitant decline in its ability to bind galactose. Notwithstanding this, the present report clearly shows that soluble, active recombinant ricin B chain can be produced in *E. coli* when the product is directed to the periplasmic space.

*Acknowledgement:* This work was supported by the Science and Engineering Research Council via grant GR/E 65296.

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