

Secretion of Mouse-Metallothionein by Engineered *E. coli* Cells in Metal-Enriched Culture Media

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Abstract

Heterologous *Escherichia coli* expression systems were designed and assayed for the synthesis of functional mouse metallothionein (MT) as a secreted fusion protein. MT secretion was compared among different systems, and the optimum vector/host/medium combination was tested for metal removal. In this case, the Cu content of the medium decreased by up to 34% after growth of recombinant bacteria. The potential use of these genetically-engineered bacteria for water bioremediation is discussed as an alternative to cytoplasmic MT or membrane-bound MT heterologous expression systems.

Excessive heavy-metal accumulation and circulation in the biosphere are important environmental and health concerns, due to the toxicity both of essential (Cu, Cr, Zn, Mn, Fe, Ni, Mo) and xenobiotic metals (Cd, Pb, Hg) at increased levels of bioavailability (Nriagu *et al.*, 1988). MT are small, cysteine-rich proteins that exhibit a high, broad-heavy metal binding capacity and extreme conformed cluster stability (Kägi, 1993). Obviously, they became the evident target for protein engineering schemes that aimed at metal chelation for bioremediation purposes. To this end, several genetic engineering approaches were developed (i) in order to increase the metal uptake of native bacteria by surface display, either of synthetic peptides or of MT molecules (Sousa *et al.*, 1996, 1998; Valls *et al.*, 1998, 2000; Mejàre *et al.*, 1998; Kotrba *et al.*, 1999 a and b); (ii) to design metal-specific bioaccumulating microorganisms (Chen *et al.*, 1997); and (iii) to synthesize recombinant mammalian MT for further biotechnological use (Brower *et al.*, 1997; Cols *et al.*, 1997; Bofill *et al.*, 1999; Pazirandeh *et al.*, 1999). However, the possibility of engineering bacteria for MT secretion, and the characterization of the corresponding protein and protein-metal aggregates have remained unexplored. Protein secretion (i.e. export to the culture medium of recombinant proteins) is the least developed and most empirical area in *E. coli* heterologous expression methodology. This is mainly due to the specialized export mechanisms underlying secretion of the few extracellular *E. coli* proteins, and to the important barrier that the outer-membrane of Gram-negative bacteria

represents (Uhlen *et al.*, 1992; Pugsley, 1993). Despite these drawbacks, and the singular structural features of MT, we successfully constructed two *E. coli* expression systems able to secrete MT as a carboxy-terminal fusion to two IgG binding domains of the *Staphylococcus* Protein A (Z domain). By means of the Protein A- or the OmpA-signal peptides, the fusion protein was successfully secreted into the culture medium, from which it could be readily purified and characterized. The decrease in copper concentration attributable to the growth of the engineered *E. coli* cells was quantified.

E. coli ZZ-MT Secretion Led by the Protein A Signal Peptide

The mouse MT-1 cDNA was amplified from the previous construct pGEX-4T-1-MT (Cols *et al.*, 1997) by PCR and subcloned into the expression vector pEZZ-18 (Amersham-Pharmacia) to yield the recombinant plasmid pEZZ-MT. pEZZ-18 contains several regions derived from the *Staphylococcus Protein A* gene that were proven to be functional in *E. coli* cells: the constitutive promoter and sequence encoding for the signal peptide and two Z domains (Nilsson *et al.*, 1990; Uhlen *et al.*, 1990). To assess ZZ-MT secretion, *E. coli* HB101 cells (*sup* E44 *hsdS20 recA13 ara-14 proA₂ lacY1 galH2 rpsL20 xyl-5, intl-1*) transformed with pEZZ-MT were grown in 20 ml of complete Luria-Bertani (LB) medium, supplemented with 100 µg/ml of ampicillin and 300 µM ZnCl₂, for 16 hours at 37°C. After growth, the medium was freed from cells by centrifugation at 3,500 x *g* in order to analyze the secreted products. The preparation was concentrated to one tenth of the initial volume by use of 3kDa Centriprep (Amicon) membranes. From the same experiment, periplasmic proteins were obtained by the osmotic shock of pelleted cells (Nilsson *et al.*, 1990), and the remaining spheroplasts were sonicated in PBS (phosphate-buffered saline, 10mM sodium phosphate (pH 7.4), 150 mM NaCl, 3mM KCl) in order to extract soluble cytoplasmic proteins. Preparations from the three compartments were electrophoresed on SDS-12.5% polyacrylamide gels (PAGE) (Laemmli, 1970), which were stained with Coomassie Blue R-250. From the PAGE results, it was clear that transformed cells synthesized and secreted a protein of the size expected for the processed ZZ-MT fusion, i.e. 20.7 kDa (data not shown). When the culture medium obtained from the previous experiment was fractionated through IgG-Sepharose affinity chromatography, the reported 20.7 kDa-protein was recovered after elution with 0.5 M AcOH (pH 3.4), indicating that the major product secreted by recombinant cells corresponded to the ZZ-MT fusion. These results confirmed the feasibility of the secretion of an engineered MT by *E. coli* cells. However, two main drawbacks prompted us to design an alternative expression system: the constitutive synthesis of the recombinant protein, and the fact that

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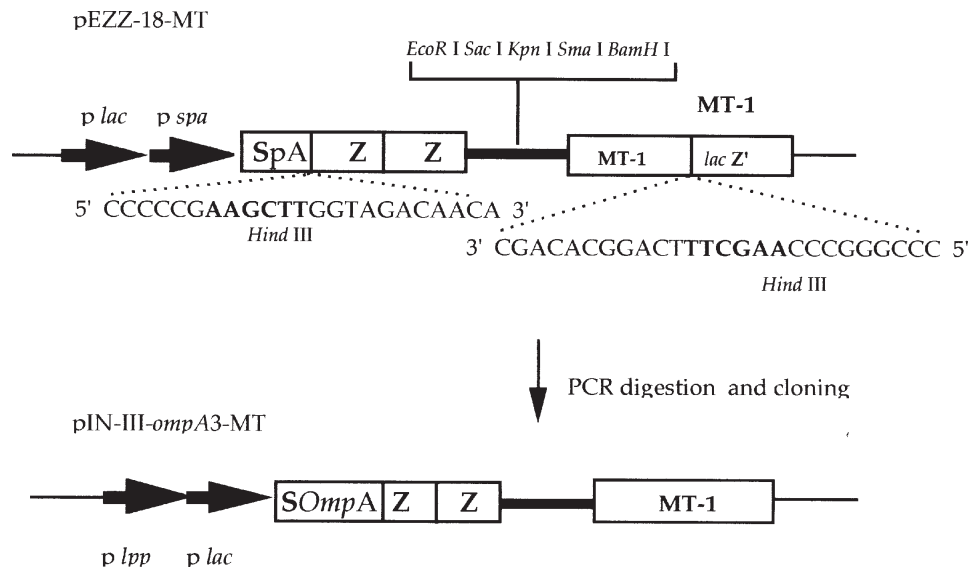


Figure 1. Cloning of the ZZ-MT-encoding construction in the pIN-III-OmpA₃ expression vector. To construct the pEZZ-18-MT plasmid, the mouse MT-1 cDNA had previously been amplified from pGEX-4T-1-MT (Cols *et al.*, 1997) using the 5'CCCCGGATCCTATGGACCCCAACTGC3' (upstream) and 5'CCCCGGGCCCAAGCTT TCAGGCACAGC3' (downstream) primers to generate *Bam*HI and *Hind*III flanking restriction sites. After digestion with these enzymes, the cDNA fragment was inserted into the pEZZ-18 vector. In order to generate the pIN-III-ompA₃-MT plasmid, the region encompassing the ZZ-MT construct was amplified from pEZZ-18, using the specific oligonucleotides shown in the figure, which generated flanking *Hind*III restriction sites. The *Hind*III-digested amplified fragment was subsequently subcloned into the pIN-III-ompA₃ vector. Restriction sites in the oligonucleotide sequences are indicated by bold type.

translocation of ZZ-MT across the outer membrane could not be reproduced in larger scale (fermentator) cultures, in which the peptide systematically remained within the periplasmic space.

E. coli ZZ-MT Secretion Led by the OmpA Signal Peptide

Plasmid pIN-III-OmpA₃ (Ghrayeb *et al.*, 1984) contains an isopropyl-1-thio-β-D-galactopyranoside (IPTG) inducible

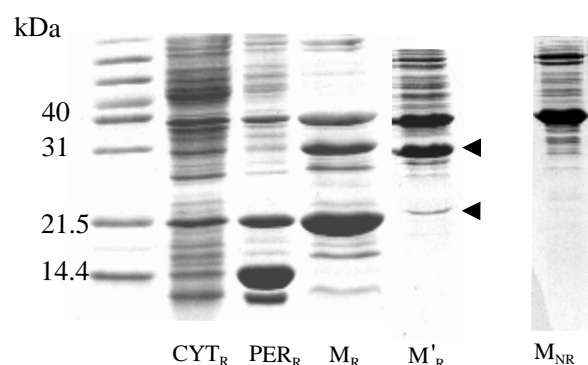


Figure 2. Localization of the ZZ-MT fusion in protein extracts and culture medium of pIN-III-ompA₃-MT transformed BL21 cells (R) and non-recombinant controls (NR). Overnight cultures were diluted 1/10 in fresh medium (20 ml) containing 100 μg/ml ampicillin, induced after 2 h with 1 mM IPTG, supplemented with 50 μM ZnCl₂ after an additional half hour and grown for a further 18 h. The culture medium was subsequently recovered, as were periplasm and cytoplasmic proteins; cells were fractionated. Proteins were detected in Coomassie Blue-stained SDS-PAGE gels. CYT: cytoplasm; PER: periplasm; M: culture medium, tenfold concentrated; M': medium in a replica assay. M_{NR}: culture medium of non-recombinant cells. The location of the ZZ-MT fusion is indicated by arrows.

lpp-lac promoter, controlling the expression of cloned cDNA fused in frame to the OmpA-signal-peptide encoding sequence. Although initially designed for transport to periplasm (Hsiung *et al.*, 1986), leading secretion of recombinant small proteins has been shown to be useful (Molina *et al.*, 1992). To assay MT secretion in this system, the region coding for the ZZ-MT fusion was PCR-amplified from the previous pEZZ-MT construct and ligated into pIN-III-OmpA₃, rendering the recombinant pIN-III-OmpA-MT plasmid (Figure 1). Correct insertion orientation was first verified by restriction analysis, positive clones subsequently being subjected to DNA sequencing (ABI377 Applied Biosystems Automatic Sequencer). *E. coli* JM105 (*thi rpsL endA sbcB15 hasdR4 Δ[lac-proAB] F'[traD36 proAB lac^l lacΔM15]*) was used for cloning procedures, while five additional strains were transformed with pIN-III-ompA-MT for expression assays: HB101, the same strain previously used with the pEZZ-MT plasmid; the protease deficient strain BL21 (*F⁻ dcm ompT_{rB}-mB₅-lon*); and the three following isogenic strains: UT2300 (*F⁻ ara-14 leu-B6 azi-6 lacY1 proC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1*), UT5600 (UT2300 Δ *ompT-fepC266*) (Elish *et al.*, 1988) and JK321 (Δ *ompT proC leu-6 trpE38 entA zih12::Tn10 dsbA::kan*) (Jose *et al.*, 1996). Different culture media - LB, MJS (12.5 mM HEPES pH 7.1, 50 mM NaCl, 20 mM NH₄Cl, 1 mM KCl, 1 mM Mg Cl₂, 0.1 mM CaCl₂, 0.05 mM Mn Cl₂, 0.8% (wt/vol) casaminoacids, and 0.4% (vol/vol) glycerol) and M9CAS (6 mg/ml Na₂PO₄, 3 mg/ml KH₂PO₄, 1 mg/ml NH₄, 0.5 mg/ml NaCl, 0.24 mg/ml MgSO₄, 0.01 mg/ml CaCl₂, 0.001 mg/ml thiamine, 2 mg/ml casaminoacids, 0.2% glycerol)- were also assayed, due to the dependence of protein secretion upon culture conditions (Molina *et al.*, 1992; Agrwal *et al.*, 1993) and to the precipitation sometimes observed when metal salts

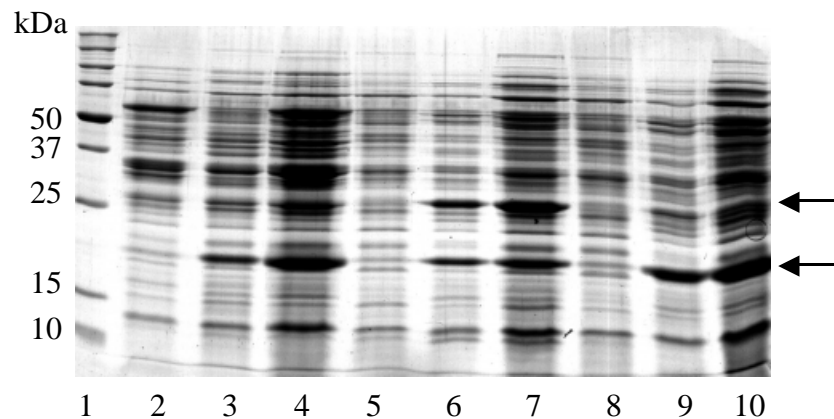


Figure 3. SDS-PAGE analysis of the proteins secreted to the culture medium (20 ml) by three isogenic strains transformed with pIN-III-*ompA*-MT: lanes 2 to 4: JK321 (*OmpT*⁻ *DsbA*⁻); lanes 5 to 7: UT5600 (*OmpT*⁻ *DsbA*⁺); and lanes 8 to 10: UT2300 (*OmpT*⁺ *DsbA*⁺). Lane 1: molecular weight markers. Lanes 2, 5 and 8: before induction (t=0 h); lanes 3, 6 and 9: t=4 h. after induction; lanes 4, 7 and 10: t=18 h. after induction. The location of the ZZ-MT fusion is indicated by arrows.

were added. In this set of experiments, overnight cultures were diluted 1/10 in 20 ml of fresh medium containing 100 µg/ml ampicillin, induced after 2 h with 1mM IPTG, supplemented with ZnCl₂ or CuSO₄ at 50 or 150 µM after an additional half hour, and grown for a further 18 h. Secreted, periplasmic and cytoplasmic protein extracts from three time lapses - 0 h, 4 h and 18 h - were prepared and analyzed as described for the pEZZ system.

HB101 and BL21 were the first strains assayed for ZZ-MT secretion in this system. Among all medium/strain combinations, a secreted product of 20.7 kDa, presumably ZZ-MT, was observed when transformed BL21 cells were grown in M9CAS medium (Figure 2, lane M_R). Conversely, in HB101 transformants, the recombinant protein remained intracellular, despite the positive results previously obtained with the pEZZ-MT construct. A deeper analysis of ZZ-MT secretion by BL21/ZZ-MT revealed a puzzling pattern, as two clear bands, not present in control cell cultures (BL21 transformed with non-recombinant pIN-III-*ompA*, Figure 2, lane M_{NR}) were always detected. One of these had the expected size for ZZ-MT, 20.7 kDa, the other corresponded approximately to 31 kDa (Figure 2, lane M_R). Both proteins contained Z domains, as they were invariably co-purified by IgG affinity chromatography. Furthermore, both were recognized by goat anti-rabbit-IgG (GAR) and rabbit anti-mouse-IgG (RAM) antibodies complexed to peroxidase (Nordic, at 1:3000 dilution) in Western blots carried out after protein electroblotting to polyvinylidene difluoride (PVDF) membranes (Millipore) blocked with 10% skim milk/PBS and developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 3% hydrogen peroxide (data not shown). N-terminal sequencing analysis of these protein bands, isolated by SDS/PAGE separation and transferred onto PVDF membranes, was performed on an Applied Biosystems Procise Sequencer. Results confirmed that in both cases the protein was ZZ-MT, from which the *OmpA* signal peptide had been correctly processed. Analysis of the periplasm/cytoplasm protein extracts of the BL21 producing cells revealed that only the 20.7-kDa band was present in these compartments (Figure 2, lanes CYT_R and PER_R). Repeated secretion assays suggested an exchange between the 20.7 kDa and 31 kDa bands,

supporting the hypothesis that the major band (of unexpected size) could be due to some post-translational modification of the ZZ-MT protein possibly related to its release into the supernatant environment. Although the minor band was more often predominant, in some cases the 31 kDa product was unexpectedly favoured (Figure 2, lane M_R). On the assumption that both secretion products shared the same composition, we made many attempts to find experimental conditions that would influence the recovery of ZZ-MT as two distinctly migrating forms. Increasing concentrations of β-mercaptoethanol and SDS in the sample buffer, addition of urea 8M in the media preparations, and PAGE separations in non-denaturing conditions were assayed, but always with negative results. Gel filtration FPLC chromatography (Superdex-75, Amersham-Pharmacia) was equally unsuccessful, as both molecules invariably co-eluted in the same fractions.

The high cysteine content of MT led us to the hypothesis that the 31 kDa band could correspond to oxidized ZZ-MT peptides formed by intramolecular disulfide bridging during the periplasm/outer membrane step, and which could not be stable in the presence of all the denaturing agents assayed. To shed light onto this subject, the influence of DsbA, the major *E. coli* disulfide bond-forming enzyme, was investigated by transformation of the *OmpT*⁻ *DsbA*⁺ strain JK321 with pIN-III-*ompA*-MT. Controls were the *OmpT*⁻ *DsbA*⁺ and *OmpT*⁺ *DsbA*⁺ isogenic strains, UT5600 and UT2300, respectively. Secretion of ZZ-MT was obtained with all the strains except for HB101, probably due to certain genetic background incompatibility. The 20.7 kDa-band was invariably detected, but the presence and relative significance of the 31 kDa-band was clearly strain-dependant (Figure 3). We were able to draw interesting observations from these results. First, that the expected ZZ-MT form (20.7 kDa) is secreted in the UT2300 genetic background independently of the *OmpT*/*DsbA* host cell genotype (Figure 3, lanes 3, 4, 6, 7, 9 and 10). Second, that the 31-kDa form appears as a secretion by-product only in the *DsbA*⁺ cells (Figure 3, lanes 6, 7, and 9), which are able to catalyze disulfide bond formation, and therefore that this form is probably due to an oxidized conformation of ZZ-MT. Finally, that in *OmpT*⁺ cells, the unexpected 31

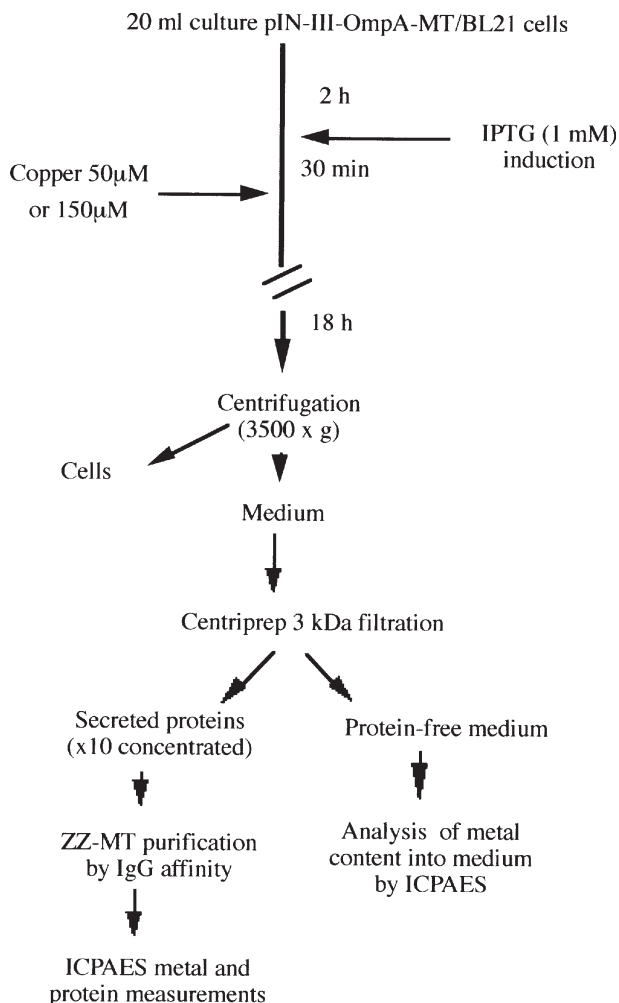


Figure 4. Diagram of the protocol for copper removal evaluation and ZZ-MT purification and characterization from pIN-III-*ompA*-MT/BL21 cultures.

kDa band is rather faint, almost disappearing after 18 h. of culture.

As DsbA is a periplasmic enzyme, it might be assumed that the 31-kDa band was formed by disulfide bridging of cysteines not involved in metal coordination in the ZZ-MT complexes, and that the absence of DsbA would prevent this oxidative reaction. Nevertheless, if some Cys residues remained metal-free, their oxidation would be immediate in the extracellular oxygen-rich environment, once secreted by any host cell, and the 31-kDa band would also appear.

As this is not the case, a more plausible explanation is that the MT portion of ZZ-MT is fully metalated inside the cell, and that the DsbA periplasmic activity on the metal-MT complexes causes the release of metal ions and the subsequent disulfide bridge formation. In support of this hypothesis, it should be noted that this reaction has already been shown to proceed *in vitro* (Maret *et al.*, 1998). Additionally, the presence of the 31-kDa band in DsbA⁺ cells is dependent on the OmpT activity of their outer cell membrane. In the two OmpT-deficient cells (BL21, UT5600) the absence of the proteolytic activity would prevent degradation of both recombinant proteins. Conversely, in the OmpT⁺ background, the 31-kDa form appears readily proteolyzed. The lack of a compact metal-MT structure would easily account for the differential degradation of the 31 kDa vs. 20.7 kDa band, as it is fully accepted that metal aggregation renders the MT molecules resistant to protease degradation (Winge and Miklosy, 1982). Furthermore, the ZZ-MT sequence includes three basic-basic amino acid pairs, which constitute the cleavage substrates for the OmpT protease, largely associated with the degradation of recombinant peptides produced in *E. coli* (Kramer *et al.*, 2000).

The Metal Binding Functionality of Secreted ZZ-MT Fusion. Copper Removal Studies

Copper (as CuSO₄ salt) was the metal most readily dissolved in the M9CAS medium, which prompted us to consider its use in the quantification of the metal removal capacity of the pIN-III-*ompA*-MT/BL21 system. To this end, 20 ml cultures of pIN-III-*ompA*-MT/BL21, as well as controls for BL21 cells transformed with the non-recombinant plasmid, were processed following the protocol shown in Figure 4. Special care was taken to prevent non-specific metal binding to the IgG Sepharose matrix. OD₆₀₀ measurements confirmed an equivalent growth rate for both cultures. Inductively-Coupled-Plasma-Absorption-Emission-Spectrometry (ICPAES) measurements were carried out in Thermo Jarrell Ash, Polyscan 61E, at 182.0 nm for S and 324.7 nm for Cu (Bongers *et al.*, 1988). The sulfur content of the recovered protein indicated a yield of ZZ-MT secretion of 8 mg/l culture. Copper results indicated that cells secreting recombinant ZZ-MT caused a decrease of 18% and 34.4% of the initial copper content of medium supplemented with 50 μM and 150 μM Cu²⁺, respectively (Table 1). Growth of control cells invariably decreased the initial copper concentration by 5%, possibly due to the inherent metabolic uptake of this metal. Measurements of copper bound to the purified ZZ-MT protein (Table 1) confirmed that

Table 1. Copper removal by pIN-III-*ompA*-MT-transformed BL21 cells, expressing ZZ-MT peptide, after 18 hours of growth in M9CAS media containing 50 or 150 μM CuSO₄, and control non-recombinant cells grown in the same conditions.

Copper added	Metal removed		Copper bound to ZZ-MT
	Control cells	Cells with ZZ-MT	
50 μM	5.1% (127 μg/l)	18% (470.8 μg/l)	3 Cu(I) ions/molecule
150 μM	5.64% (444 μg/l)	34% (2679 μg/l)	9 Cu(I) ions/molecule

synthesis and secretion of the recombinant protein were responsible for the decrease in copper concentration. These results are consistent with Cu-MT aggregation taking place inside the secreting cells, rather than in the medium, since copper binds to MT as Cu(I) and the reduction of the supplemented Cu(II) to Cu(I) only proceeds inside cells. This also agrees with the removal of some metal ions for the DsbA⁺ cells in the periplasm, previously postulated, and is also consistent with the reduced efficiency of external MT membrane-bound systems reported for copper removal in comparison with cadmium or zinc removal (Sousa *et al.*, 1996; Kotrba *et al.*, 1999 a and b).

We report for the first time the successful synthesis of mammalian MT as a functional *E. coli*-secreted protein. The high cysteine-content of this peptide and the peculiar conformation of its metal clusters are both features that challenged the feasibility of this recombinant synthesis. Unlike cytoplasmic-MT or membrane-bound-MT synthesising bacteria, secreting micro-organisms do not become self-saturated with the recombinant protein, thus allowing non-restricted synthesis of the chelating agent. Although our results were obtained at an analytical level, and are therefore not suitable to direct biotechnological application, further studies to broaden the metal-binding capacity of this system may contribute to new bioremediation schemes.

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