Volume 14 Number 18 1986 Nucleic Acids Research

Secretion of heterologous gene products to the culture medium of *Escherichia coli*

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Received 17 June 1986; Revised and Accepted 19 August 1986

ABSTRACT

Different constructs containing fragments of the Staphylococcal protein A gene have been introduced in *Escherichia coli* and the effect on expression and translocation of the various heterologous gene products have been studied. By reversing the orientation of the different protein A gene constructions in the plasmid vector, a dramatic 20-fold difference in expression was obtained, accompanied with secretion of the gene product to the culture medium. Similar results were obtained by "heat-shock" treatment of the *E.coli* host cells. These results suggest the presence in the protein A gene of a stress induced promoter, functional in *E.coli*. The system was used to efficiently secrete a fusion protein consisting of a protein A fragment and human insulin-like growth factor I (IGF-I) to the culture medium of *E.coli* HB101. The fusion protein was purified from the culture medium by IgG affinity chromatography in a one-step procedure giving more than 95% yield.

INTRODUCTION

Expression of heterologous proteins in prokaryotic hosts has faced several problems (1,2). Often the gene product is unstable in the host bacteria and thus becomes rapidly degraded. It is also common with incorrect folding giving biologically non-active molecules. This problem has frequently been observed with peptides which are dependent on a correct formation of disulfide bridges, that cannot be formed due to the reducing environment in the cytoplasm of most bacteria (2). To stabilize heterologous peptides in *Escherichia coli*, gene fusions involving the *lac*Z gene have been used, e.g. for expression of somatostatin (3) and insulin (4). *In vivo* such hybrid proteins often precipitate intracellularly and form so called inclusion bodies (5). After the solubilization procedure and purification, the proteins are often in a non-active form, and complex renaturation procedures must follow.

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An alternative strategy is to secrete the gene product out of the reducing environment of the cytoplasm. A correct folding of peptides containing disulphide bridges may then be achieved. Secretion also has the advantage of simplifying the recovery of the product, in particular when excretion to the culture medium is obtained. Gram positive bacteria, such as _Bacillus subtilis_, _Staphylococcus aureus_ and _Streptomyces lividans_ which effiently secrete many proteins into the medium, have therefore been used extensively to develop host-vector systems designed to express and secrete recombinant products (6,7,8). Similar systems have also been developed for _E.coli_ although the product has to be recovered from the periplasmic space rather than from the culture medium (9).

In order to obtain truly excreting _E.coli_ host-vector systems, in which the gene product is transported out of the cell, several approaches have been tried. First, leaky (_lky_ or _exc_) mutants of _E.coli_ K12 have been isolated and used for optimized extracellular production of alkaline phosphatase (10) and beta-lactamase (11). Second, secretion vectors based on genes encoding _E.coli_ outer membrane proteins have been constructed (12). Finally, genes encoding normally extracellular proteins from Gram-positive bacteria have been used to construct expression vectors for _E.coli_ (13). Although some of these systems have intereresting potential, they have not yet proved to be a general system for the production of heterologous proteins.

Recently, we reported (14) that a small fragment of the staphylococcal protein A gene encoding region E followed by B fused to the signal sequence, gave a correctly processed and efficiently secreted product in _E.coli_. Unexpectedly, the gene product EB, was excreted to the culture medium, a phenomenon accompanied by dramatic morphological changes of the _E.coli_ cells. It was suggested (14) that the outer membrane was affected, thereby allowing passage of proteins which are usually found only in the periplasm. Although the protein A promoter is constitutively expressed during growth, the changed morphology did not seriously affect the viability of the cells. Little cell lysis was detected but the generation time during growth was slightly longer than normal.

In this report, we have analyzed this phenomenon in more detail and also used the system to express and secrete heterologous proteins to the culture medium of E.coli HB101. A fusion protein consisting of fragment E repeated twice and human insulin-like growth factor I (IGF-I) was produced and purified from the culture medium by IgG affinity chromatography making use of the binding of fragment E to IgG.

MATERIAL AND METHODS

Bacterial strains and plasmids

E.coli HB101 was used as bacterial host (15). Plasmids pRIT4 (16), pASE3(14), pASEE (17), pEMBL8 and pEMBL9 (18) were used as indicated.

DNA constructions

Restriction enzymes (Pharmacia, New England Biolabs and Boehringer Mannheim) and T4 DNA ligase (New England Biolabs) were used according to the suppliers′ recommendations. Transformation and growth of E.coli were performed as described earlier (19).

The plasmid pASEE (17) contains the staphylococcal protein A promoter, signal sequence and region E fused to another region E with a dodecameric EcoRI linker between the two E regions. Downstream of the second E region there is a mp8 linker (16) from EcoRI to HindIII. A synthetic gene encoding human insulin-like growth factor I (IGF-I) was inserted after this second region E. The synthesis and cloning of the IGF-1 gene has been described earlier (22). The plasmid pASEE was cleaved partially with EcoRI and the linearized vector was isolated from an agarose gel after electrophoresis. This isolated fragment was cleaved with HindIII and subsequently recovered from an agarose gel. This fragment was ligated to an isolated IGF-1 gene contained within a 240 bp EcoRI/HindIII fragment.

Colonies harboring the correct plasmid were distinguished from those containing only one region E fused to IGF-I due to the divalent binding of EE to IgG. When secreted this gives halos of precipitate around the colony on canine serum agar plates. The resulting plasmid, pRIT14, contains the protein A promoter and signal sequence in front of the gene fusion EE-IGF-I.

By reversing the orientation of this construct plasmid pRIT15 was obtained (Fig 1). The plasmid pRIT14 was digested with TaqI and HindIII and the DNA fragment containing the protein A promoter and signal sequence in front of the gene fusion EE-IGF-I was recovered from an agarose gel. This fragment was inserted into the plasmid pEMBL8, previously digested with AccI and HindIII. This gave the plasmid pRIT15 with the gene fusion in the reverse orientation as compared to pRIT14 (Fig 1).

Protein preparations and assays

Cell extracts were made as described earlier (19). An osmotic shock procedure was used to release protein from the periplasmic space (20). IgG Fast Flow Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography was used to purify and concentrate protein A and protein A derivatives. The fusion protein was cleaved with hydroxylamine as described by Bornstein and Balian (21). Detection and quantification of protein A was performed by ELISA using Fab fragment of rabbit anti-protein A antibodies conjugated to beta-galactosidase (14), a kind gift from Dr M Inganäs (Pharmacia, Sweden).The analysis by SDS-polyacrylamide gel electrophoresis was performed as described earlier (17).

RESULTS

Construction of various gene fusions

We have recently subcloned and expressed two of the IgG-binding regions (E and B) of staphylococcal protein A (17). Using secretion vectors (14), it was possible to obtain translocation of these fragments, and several combinations of them, to the culture medium of E.coli HB101. However, both yield and efficiency of secretion varied between different constructs. A more than 80-fold difference in the amount of gene product was obtained and the fraction excreted to the culture medium varied between 5 and 95 percent (17).

To investigate the factors involved in these dramatic variations in yield and translocation efficiency, we decided to express different protein A derivatives using several vector systems. Basically, the protein A gene fragments were inserted in both orientations into similar high copy number plasmids. In

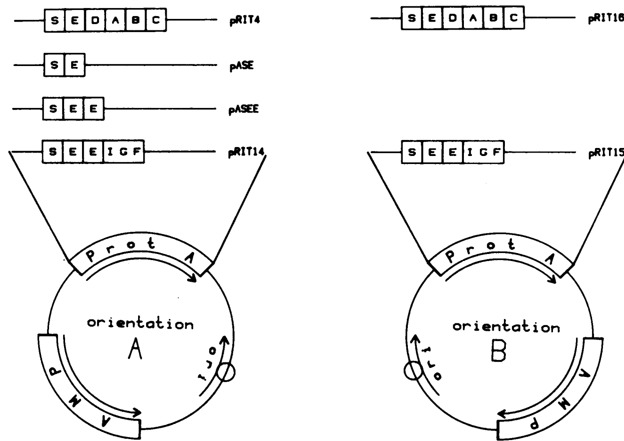

Fig 1. Schematic drawing of the different gene constructs. The protein A gene consists of the signal sequence (S) and five IgG-binding regions (E,D,A,B and C). The synthetic DNA fragment encodes the human insulin-like growth factor I (IGF). The orientations reflect the direction of the protein A transcription in relation to the transcription of the ampicillin gene.

this way, transcription of the protein A gene fragments proceed either in the opposite (orientation A) or in the same (orientation B) direction as the transcription of the ampicillin gene and the replication of the host plasmids.

Essentially, three different types of gene fusions were assembled; (i) plasmids pRIT4 and pRIT16 containing the whole IgG-binding part of staphylococcal protein A, (ii) plasmids pASE and pASEE containing one and two IgG-binding fragments, respectively and (iii) plasmids pRIT14 and pRIT15 encoding a fusion between two IgG-binding fragments and the human insulin-like growth factor I (IGF-I). The latter constructions were used to elucidate if the expression-secretion system can be used for the expression of foreign gene products into the culture medium of E.coli. The various constructs are schematically shown in Fig 1.

The synthesis and cloning of the gene encoding human IGF-I has previously been described (22). The gene is contained in a 240 base pair EcoRI/HindIII fragment with the start codon ATG a few base pairs downstream from the EcoRI site. We have earlier

described a novel approach to use oligonucleotide mediated *in vitro* mutagenesis whenever a restriction site is close to the point of mutagenesis (19). Using the same protocol, the ATG start codon was changed to AAT thus changing the N-terminal methionine residue of the IGF-I gene into an asparagine residue (E Holmgren and B Nilsson, manuscript in preparation). This generated a dipeptide sequence asparagine-glycine permitting cleavage with hydroxylamine which hydrolyzes the peptide bond between these two residues (21). By fusing this new IGF-I fragment to a protein A fragment, efficient affinity purification can be obtained. Furthermore, IGF-I molecules containing the native N-terminal glycine residue, can be produced by the chemical treatment.

We have earlier described the assembly of plasmids pRIT4 (16), pASE (14) and pASEE (17). Plasmid pRIT16 was obtained from pRIT4 by inserting the protein A fragment in the reverse orientation. The gene fusion between fragments EE and IGF-I was obtained by inserting the synthetic IGF-I gene into the vector pASEE (see Materials and Methods for details).

Expression of the different constructs in E.coli

E.coli HB101 containing the plasmids shown in Fig 1, were grown overnight and the amount of protein A in the different cellular compartments was determined using anti-protein A antibodies (14). The results presented in Table 1, show that plasmid pRIT4 gives relatively low levels of expression (2 mg/l) of protein A. As expected, most of the gene product is found in the periplasm. Interestingly, the same construct in the opposition orientation, pRIT16, gives 20-fold higher expression and most of the protein A molecules are exported to the culture medium. Thus dramatic differences can be obtained simply by reversing the orientation of the protein A gene. This was also accompanied by filamentous growth of the *E.coli* host cells, a phenomenon previously observed for the expression of smaller protein A fragments (14).

The next set of expression studies involved the plasmids pASE and pASEE encoding one or two IgG-binding domains, respectively (17). As shown in Table 1 both plasmids confer filamentous growth to their host cells and the protein A

Table 1 Expression and localization of various protein A derivatives in E.coli cells containing different plasmids.

Plasmid	Vector type	Product	Filamentous growth	Expr. level (mg/l)	Intra-cell. (%)	Peri-plasm. (%)	Extra-cell. (%)
pRIT4	A	EDABC	(-)	2	2	89	9
pRIT16	B	EDABC	++	41	3	28	69
pASE	A	E	+	0.2	1	5	94
pASEE	A	EE	+	33	1	24	75
pRIT14	A	EE-IGF	+	31	2	21	77
pRIT15	B	EE-IGF	++	61	2	13	85

E.coli HB101 containing the different plasmids were grown over night and the cells were treated by an osmotic shock procedure (periplasm) followed by sonication of the osmotically shocked cells (intracellular). Detection and quantification of protein A was performed by ELISA as described before (14). Filamentous growth indicates the morphology of the host cells as observed by light microscopy. Vector type refers to the orientation as shown in fig. 1.

fragments are efficiently secreted to the culture medium, 94 and 75 percent, respectively. However, the amount of divalent fragment EE produced is more than 80-fold higher than for the monovalent fragment E. In each case, identical promoter, signal sequence and host plasmid are used (17). The reason for this dramatic difference in yield is unknown, but fragment E may be relatively unstable, before or after it is released to the culture medium of E.coli. Therefore, fusion vectors based on truncated protein A should contain two IgG-binding fragments, in order to obtain good yields as well as efficient secretion to the culture medium.

Expression studies using plasmid pRIT14, encoding the divalent fragment EE fused to human IGF-I, support this conclusion. The same pattern of filamentous growth, good yields and efficient secretion is observed (Table 1) despite the fact that a human peptide hormone has been fused to the C-terminal end of fragment EE. It is interesting that the reversed orientation of the protein A IGF-I gene fusion (pRIT15) gives a higher level of expression as well as more efficient secretion.

Table 2 Expression and localization of protein A in E.coli cells containing plasmid pRIT4 grown at different temperatures

Temperature	Absorbance (580 nm)	Level of expression (mg/1)	Filamenous growth	Extra-cellular (%)
30°C	0.21	0.16	(-)	13
42°C	0.29	3.14	+	68

E.coli HB101 containing plasmid pRIT4 were grown at 30°C to an absorbance of 0.05 (580 nm) and then grown at 30°C or 42°C for 3 hours. Quantification and localization of protein A as well as cellular morphology was determined as described in Table 1.

This is similar to the effect on protein A expression (pRIT4 versus pRIT16) but the effect is less dramatic.

Heat induction of expression and secretion

The expression studies presented in Table 1 show that the translocation of the protein A derivatives to the growth medium of the host cells were always accompanied by filamentous growth. On the other hand, E.coli cells containing plasmid pRIT4 retain most of the gene product in the periplasm and did not show a changed morphology. The question arises whether the observed phenomenon with the other protein A derivates is due to a stress-response of the host cells. In this case, it might be possible to induce the phenotypic alterations of cells containing pRIT4 simply by treatments known to cause stress-response in E.coli. However, no clear effect was observed by treating the cells with agents, such as nalidixic acid, which are known to induce an "SOS"-response in E.coli (data not shown). Instead "heat-shock" treatment was performed. E.coli HB101 containing pRIT4 was grown at 42°C for a few hours and the amount and localization of protein A was determined. A comparison with cells grown at 30°C is presented in Table 2 and reveals a clear heat-induced response. The cells grown at 42°C are filamentous, produce 20-fold higher amounts of protein A compared to the cells grown at 30°C, and the gene product becomes predominantely extracellular. Clearly, the lack of phenotypic changes of E.coli cells containing pRIT4, as compared to the other plasmids of Table 1, can be counteracted by heat treatment of the cells.

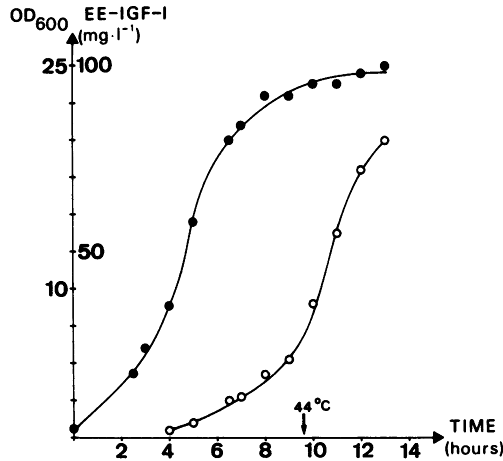

Fig 2. Growth curve of E.coli HB101 containing plasmid pRIT15 and the amount of fusion protein EE-IGF-I in the culture medium. The cells were grown to early stationary phase at 37°C followed by a temperature shift to 44°C (arrow). At different times the OD at 660 nm was measured (filled circles) and the amount of fusion protein in the culture medium determined in an ELISA (open circles).

Expression, secretion and purification of the fusion protein

In order to evaluate if human IGF-I can be produced and purifed using the divalent fragment EE fusion, fermentations in a 7-liter scale was performed. E.coli cells containing plasmid pRIT15 (Fig 1) were grown in rich media using standard techniques. In figure 2 the growth curve and the amount of fusion protein EE-IGF-I in the culture medium are presented. It is noteworthy that a four hour "heat-shock" treatment yields a fourfold increase in gene product. The cell suspension was cooled to 10°C in the fermentor and the cells separated from the medium in a closed system using a cross flow microfilter (Pellicon, 0.22µm, 0.5 m^2). Approximately 80% of the medium was collected and passed through a custom-designed Sepharose Fast Flow gel column (200 ml bed volume) containing covalently coupled human IgG (Affinity Chromatography Workshop, Pharmacia Fine Chemicals, Uppsala, Sweden). The flow rate was adjusted to 3 l/h (linear flow rate:150 cm/h) giving very little flow through of fusion protein (<1%). After washing, the fusion protein was eluted using 0.5 M HAC, pH 2.8, and the collected

material was lyophilized. The overall yield for the fast flow affinity column and subsequent steps was more than 95%. Analysis by SDS-polyacrylamide gel electrophoresis revealed a major band corresponding to full-length fusion protein (not shown). As earlier observed when IGF-I fusion proteins were expressed in E.coli (19), also smaller sized proteins corresponding to proteolytic degradation products could be observed.

The purified fusion protein was treated with hydroxylamine to cleave the aspargine-glycine peptide bond (21) and further purified by ion exchange chromatography (M.Billich and T.Moks, manuscript in preparation). The specific IGF-I activity of the final product as determined by a radio receptor assay (19) was found to be approximately 10 000 U/mg (data not shown), which is the same as the value for the native hormone purified from human serum (24).

DISCUSSION

There are several reasons to develop bacterial expression systems aimed at providing heterologous production of secreted proteins. The advantages with such secretion systems compared to those giving intracellular products include: (i) no need to disintegrate the cells, (ii) an initial purification step in which all the intracellulat proteins can be removed simply by centrifugation or microfiltration (iii) an oxidative environment which allows disulfide bridges to form. So far, efforts to develop secretion vectors have been focused primarily on Gram positive hosts and attempts in Gram negative bacteria have been limited (1). E. coli, by far the most preferred host for the expression of foreign proteins, usually does not secrete proteins into the culture medium. Therefore, the observation by Abrahmsen et al. (14), that several protein A fragments can be translocated to the culture medium of the E.coli host cells is very interesting. We have now analyzed this phenomenon further with the aim of developing a general system for secretion of proteins in E. coli.

The unexpected observation (Table 1 and Figure 1) that a simple reversion of the orientation of a gene construct encoding a truncated protein A molecule gives a 20-fold higher expression

Table 3 Comparision of the proposed protein A heat shock promoter (26) with known heat shock promoters from *E.coli* (27).

Gene	"-35"	distance (bp)	"-10"
protein A	TTTACTTCCTGAA	14	AAATATTT
groE	TTTCCCCCTTGAA	13	CCCCATTT
dnaK P1	TCTCCCCCTTGAT	14	CCCCATTT
dnaK P2	TTGGGCAGTTGAA	13	CCCCTATT
C62.5	GCTCTCGCTTGAA	15	CCCCATCT
rpoD	TGCCACCCTTGAA	15	GACGATAT
lon	TCTCGGCGTTGAA	14	CCCCATAT

Positions of the -35 and -10 regions were assigned by alignement of the promoters with one another and determination of the regions having the most matches, allowing the introduction of gaps between the -35 and -10 regions.

can have several alternative explanations. First, the mRNA stability could be different depending on the difference in the structure of the 3′-end of the transcribed mRNA molecules. This is unlikely since insertion of transcription terminators directly following the gene construct did not influence the orientation effect (data not shown). Second, the transcription rate could be influenced by additional promoters or by a changed strength of existing ones. Third, the copy number of the plasmid might be influenced by the direction of the protein A transcript. In addition, post-transcriptional factors such as translation efficiency or changed stability of the gene product may influence the final yield.

Several observations suggest the existence of a stress induced promoter in front of the protein A gene. First, "heat-shock" treatment known to induce responses similar to stress in both bacteria and higher organisms (2) changes the normally periplasmatic protein A gene product of pRIT4 to become almost exclusively extracellular and at the same time changes the morphology of the host cells into filamentous (Table 2). Second, the appearance of the various protein A gene products in the culture medium was always accompanied by filamentous growth of the *E.coli* host cells. Although this morphological change is often coupled to an "SOS"-type stress response there are recent

data showing that "SOS" and "heat-shock" responses are linked (25). Finally, a putitative promoter similar to an E.coli "heat-shock" consensus promoter can be found upstream of the earlier proposed sigma-55 promoter of the protein A gene (Table 3). Although more work is needed to establish the existence and relative strength of this putative stress induced promoter, this hypothesis can explain the results presented in Tables 1 and 2. Thus, plasmid pRIT4 directs the expression of low levels of truncated protein A, not enough to induce a stress response of the host cell. In contrast, the reverse orientation (pRIT16) yields slightly higher expression, most likely enough to give a stress response in the host. The endogenous promoter is thereby induced and the effect becomes further pronounced. Similarly, both orientations of fragment EE fused to human IGF-I give high levels of gene product as well as filamentous growth. This suggests that the IGF-I fusion protein is transported or folded inefficiently compared to the truncated protein A (pRIT4) whereby the stress response is induced independently of the orientation. In this case, only a 2-fold difference is therefore obtained by reversing the orientation of the gene construct.

At present, very little is known about stress induced genes of Gram positive bacteria. However, heat shock promoters have been found to be highly conserved through evolution and it is therefore likely that a S.aureus promoter could be recognized by the heat-shock transcriptional factors of E.coli. The question arises what role this putative promoter might have in the pathogenicity of S.aureus.

Although the biological significance of the putative stress induced promoter is yet unknown, it is clear that the effect in E.coli on expression and translocation of protein A derivatives is very useful. Vectors based on two IgG-binding fragments (EE), have several advantages compared with the earlier described vectors which were based on the whole protein A gene (19). First and most important, efficient secretion to the E.coli culture medium is obtained. Second, the two fragments bind to IgG with lower affinity than protein A, which makes it possible to elute the fusion protein under slightly milder conditions (T.

Moks, unpublished). Third, the affinity "tail" is considerably smaller than protein A, which may be advantageous by avoiding steric hindrance in the affinity column and by facilitating the folding of some of the fusion proteins.

It is obvious that the expression/secretion system described here is potentially useful for secretion and purification of heterologous gene products.The fact that the purified and cleaved material has the same specific activity as IGF-I purified from human plasma, demonstrates that both the production and cleavage of the fusion protein have been succesful, and suggests that the three disulfide bridges were correctly formed.

Recently, we have used this host-vector system based on two IgG-binding regions of protein A to express several proteins and peptides in E.coli. The gene products were, in all cases, secreted to the culture medium and could be purified by affinity chromatography (T. Moks, unpublished).

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish National Board for Technical Development, the Swedish Natural Research Council and KabiGen AB, Stockholm, Sweden. We are grateful to Drs Arne Holmgren, Hans Jörnvall, Margareta Billich, Erik Holmgren, Staffan Josephsson and Lennart Philipson for critical comments and advice. We thank Gerd Benson for skilful secretarial help.

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