Recombinant *Escherichia coli* Strains Provide High-Level Expression of Human Interleukin-3 and Interleukin-4

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A number of expression vectors carrying the genes coding for interleukin-3 and interleukin-4 are constructed. The influence of the promoter type, the ribosome-binding site, and the number of plasmid copies on the expression of recombinant proteins and the stability of producing strains is studied. *Escherichia coli* strains providing a stable high level production of human interleukin-3 and interleukin-4 are obtained.

Key Words: *interleukin-3; interleukin-4; producing strain; stability; production level*

Biotechnological methods have made recombinant interleukins (IL) available for clinical investigations. At the present time two interleukins are undergoing clinical trials: IL-3, a cytokine displaying a broad spectrum of activities and playing an important role in the regulation of hemopoiesis and immune response, and IL-4, a cytoki- ne stimulating the proliferation of activated B and T cells and inducing IgE secretion and expression of low-affinity IgE receptors [8,9]. However, the high cost of these preparations has made it imperative to develop effective methods for the expression and production of biologically active human IL-3 and IL-4.

Previously we obtained *E. coli* strains producing human IL-3 [2] and IL-4 [1] as inclusion bodies in the cytoplasm. Protein synthesis is known to be heavily dependent on the structures of the ribosome-binding site (RBS), the stability of the corresponding mRNA, the promoter potential, and the toxicity of the recombinant protein for the microorganism.

We studied the expression of human IL-3 and IL-4 in a number of recombinant *E. coli* strains in order to select the producing strains providing the highest protein production and preserving their productivity during long-term culturing with successive passaging.

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MATERIALS AND METHODS

For the construction of expression vectors carrying the IL-4 gene we used the plasmid pKK223-3 (Pharmacia), its derivatives, and the plasmid pPTEhEGF (laboratory collection), a derivative of pUC18 plasmid.

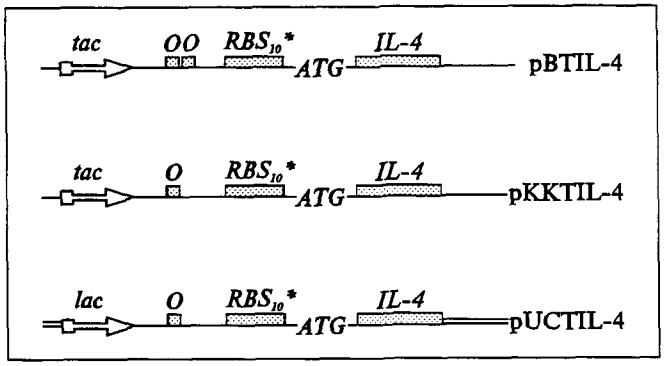

Fig. 1. Schemes of plasmids providing expression of the human IL-4 gene (pBTIL-4, pKKTIL-4, and pUCTIL-4). *tac, lac*: promoters; *O*: operator binding site for lac-repressor; RBS_{10}*: modified binding site for the ribosomes of the gene coding for protein 10 of T7 phage; *ATG*: start codon; *IL-4*: human IL-4 gene. Broken line indicates the pKK223-3 sequence. Double broken line indicates the pUC18 sequence.

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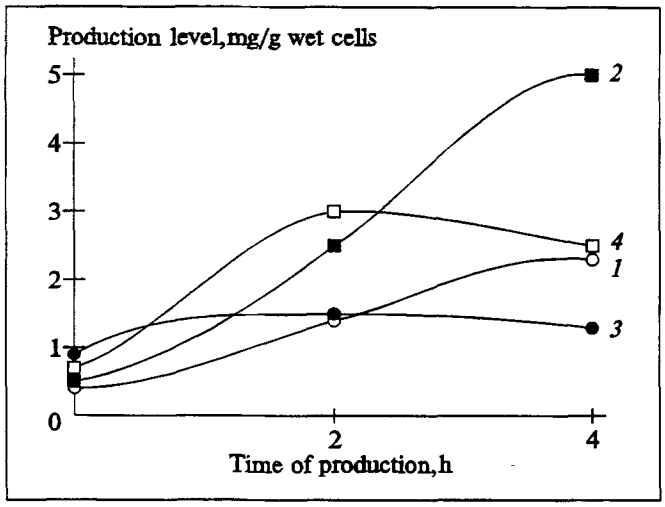

Fig. 2. Comparison of recombinant Il-4 production levels. *1*) TG1(pKKTIL-4-9) strain; *2*) TG1(pBTIL-4) strain; *3*) TG1(pKKTIL-4) strain; *4*) TG1(pUCTIL-4) strain.

The plasmid pBTIL-4 was constructed with the use of pKK223-3 as a vector in which the gene hIL-4 from the plasmid pKKhIL-4-10 [1] was cloned with a simultaneous modification of the proximal portion of the RBS sequence of gene 10 of phage T7 (the structure of the proximal region before the start codon is AGGAGATAATTCATG) and replacement of the DNA fragment containing tac-promoter by the regulatory site of the plasmid pTOTE2IL-3 [2], which carries the tac-promoter and two binding sites for lacI repressor. The plasmid pKKTIL-4, which, unlike pBTIL-4 plasmid, contains only one binding site for lacI, was constructed on the basis of the plasmids pBTIL-4 and pTE2IL-3 [2]. The plasmid pUCTIL-4 was obtained with the use of PTEhEGF and the DNA fragments pBTIL-4 and pTE2IL-3.

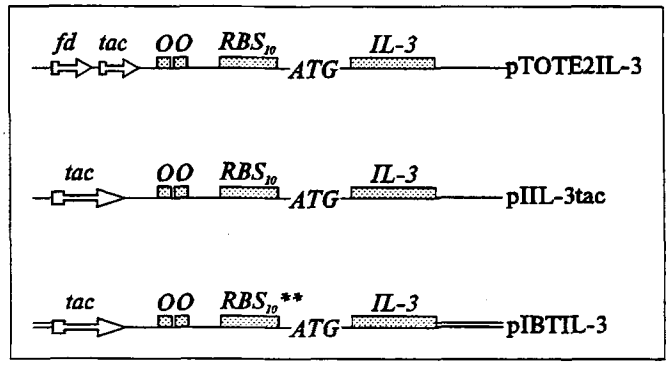

Fig. 3. Schemes of plasmids providing expression of the human IL-3 gene (pTOTE2IL-3, pIIL-3tac, and pIBTIL-3). *tac, fd*: promoters; *O*: operator binding site for lac-repressor; RBS_{10}: binding site for the ribosomes of the gene coding for protein 10 of T7 phage; RBS_{10}^{**}: modified binding site for the ribosomes of the gene coding for protein 10 of T7 phage; *ATG*: start codon; *IL-3*: human IL-3 gene. Broken line indicates the pKK223-3 sequence, double broken line indicates the pBR322 (from 2222 to 4363 nucleotide pairs) sequence.

The vectors carrying the IL-3 gene were constructed with the use of the plasmids pTOTE2IL-3 [2] and pBTIL-4.

The plasmid pIIL-3tac was obtained by removing the DNA fragment carrying the promoter of phage fd from the plasmid pTOTE2IL-3. The plasmid pIBTIL-3 was constructed on the basis of pBTIL-4 in which the IL-4 gene was replaced by the IL-3 gene. The replacement was performed with the use of synthetic oligonucleotides, which provided the following structure of the proximal region of the RBS gene 10 of T7 phage: AGGAGATATATCCATG. Standard methods [3] were employed for all genetic engineering procedures.

E. coli strain TG1 was transformed with the obtained vectors [3]. The recombinant strains were grown at 37°C in a rich medium (L-broth) in the presence of 50 mg/ml ampicillin. The synthesis of recombinant proteins was induced by the addition of 1 mM isopropylthiogalactoside to the cultures (light absorbance at 600 nm is 1 relative unit). Protein production was analyzed after two and four hours of incubation by densitometry and immunoenzyme assay [5]. The protein preparations were electrophoresed in polyacrylamide gel (PAAG) in the presence of sodium dodecylsulfate.

RESULTS

The recombinant strain TG1(pKKhIL-4-9) [1] produces IL-4 at a level of about 2 mg per gram wet cells, as evidenced by densitometry and immunoenzyme assay. The vectors pBTIL-4, pKKTIL-4, and pUCTIL-4 (Fig. 1) carry more efficient RBS, the distal region of which provides for the stabilization of the corresponding mRNA in the cells [10]. Besides, the plasmid pBTIL-4 contains an additional binding site for the recombinant protein which is foreign for *E. coli*. The plasmid pUCTIL-4 (the number of its copies in the cells is 3- to 4-fold higher compared with those of the other plasmids) carries a much weaker regulated lac promoter. Such a combination results in a 5- to 8-fold reduction in the transcription level for the recombinant gene compared with the plasmid pKKTIL-4 [4]. Recombinant strains carrying these plasmids were grown under conditions of recombinant protein induction, and IL-4 production was analyzed by densitometry and PAAG electrophoresis (Fig. 2).

It can be seen from Fig. 2 that the strain TG1(pBTIL-4) provides the highest level of IL-4 synthesis after 4 h of culturing: about 5 mg/g wet cells. In this case up to 90% of the cells preserve the recombinant plasmid. The IL-4 production by

the strain TG1(pUCTIL-4) reaches the maximum 2 h after induction and gradually declines during further incubation. The strain TG1(pKKTIL-4) maintains a moderate and practically unchanged level of IL-4 production over the entire observation period. However, rapid elimination and lysis of some cells occurred in this strain. After 4 h of culturing, less then 10% of cells were ampicillin-resistant.

Thus, the intense production of recombinant IL-4 in TG1(pKKTIL-4) and TG1(pUCTIL-4) leads to a fairly rapid elimination of the plasmid and even to lysis of some cells due to toxicity of the foreign recombinant protein for the cell. This results in a decrease in the total IL-4 synthesis in these strains.

Among the investigated *E. coli* strains, the TG1(pBTIL-4) strain proved to be the optimal: it produced 5 mg/g wet cells human IL-4 and preserved this productivity during long-term culturing.

The recombinant plasmid pTOTE2IL-3 [2] yields a very high level of human IL-3 production (about 25 mg/g wet cells in the TG1 strain. However, this strain is characterized by a rather high level of spontaneous constitutive production of IL-3, which may account for the insufficient stability of the strain during long-term culturing.

The plasmids pIIL-3tac and pIBTIL-3 (Fig. 3) contain only one tac promoter, instead of two promoters in the plasmid pTOTE2IL-3: P_{VIII} of fd phage and tac; moreover, the structure of the proximal fragment of RBS is different in pIBTIL-3. This plasmid is represented in an *E. coli* cell by 2- to 3-fold fewer copies than pTOTE2IL-3 and pIIL-3tac.

Removal of the phage promoter increases the stability of producing strain TG1(pIIL-3tac) compared with TG1(pTOTE2IL-3) without any decrease in the recombinant IL-3 production.

The strain TG1(pIBTIL-3) proved to be the best of the studied strains, exhibiting high-level productivity of recombinant IL-3 (Fig. 4). Modification of the proximal region of RBS with change of the distance between the Shine-Dalgarno sequence and ATG from 7 to 8 nucleotides for a simultaneous decrease in the number of plasmid copies stimulated IL-3 synthesis about 1.5-fold (up to 35-40 mg/g wet cells).

Thus, by varying the RBS structure, promoter type, number of operator sites for repressor binding, and the number of plasmid copies in the cell, we have constructed expression vectors used to generate *E. coli* strains with a stable high-level production of human IL-4 and IL-4.

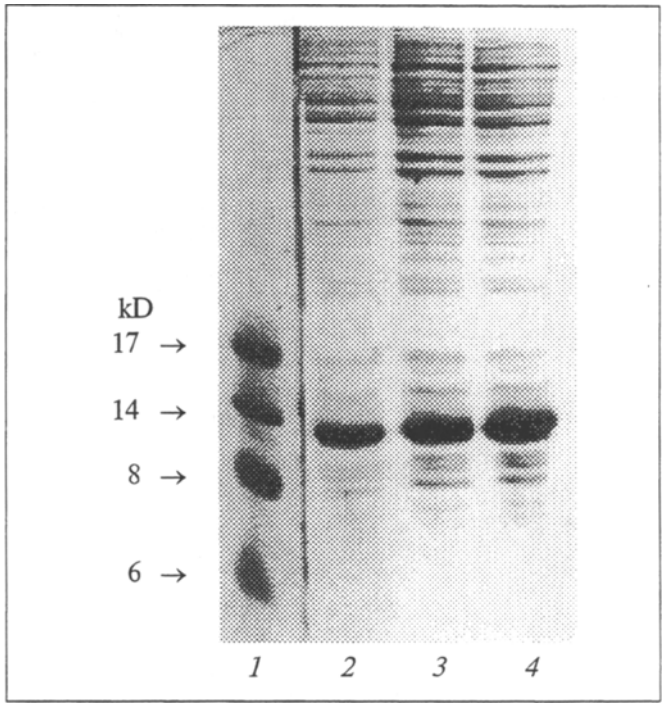

Fig. 4. Electrophoregram of the total cellular protein of *E. coli* strains TG1(pTOTE2IL−3), TG1(pIIL−3tac), and TG1(pIBTIL−3). *1*) molecular weight markers (Pharmacia); *2*) TG1(pTOTE2IL−3) strain; *3*) TG1(pIIL−3tac) strain; *4*) TG1(pIBTIL−3) strain. All the strains were grown in the presence of the inducer isopropylthiogalactoside.

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