Expression of a cloned streptavidin gene in *Escherichia coli*

(gene expression/biotin-binding protein/T7 promoter/T7 RNA polymerase)

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**ABSTRACT** We describe the construction of systems for expressing the cloned streptavidin gene in *Escherichia coli*. Although the streptavidin gene is extremely lethal to the host cells, because of the strong biotin binding of the gene product, the gene was expressed efficiently in *E. coli* by using T7 RNA polymerase/T7 promoter expression systems. The expressed streptavidin accumulated to more than 35% of the total cell protein. The expressed streptavidin was insoluble in the cell. However, after solubilization by dialysis against 6 M guanidine hydrochloride (pH 1.5) and removal of guanidine hydrochloride by dialysis, the protein became soluble and renatured. This simple procedure yielded streptavidin purified almost to homogeneity. The purified streptavidin bound 3.5–3.9 molecules of biotin per molecule, indicating that it had almost full biotin-binding ability. Some of the purified streptavidin molecules aggregated into oligomers, suggesting that the C-terminal region of the molecule, present in our material but absent in typical preparations, may be responsible for the aggregation.

Streptavidin, a protein produced by *Streptomyces avidinii*, specifically binds a water-soluble vitamin, d-biotin (1,2). The strong and specific affinity for biotin (Ki = 10⁻¹³ M) (2,3) has made streptavidin, as well as egg white avidin, a frequently used protein for detection and characterization of various biological substances (3–7). Streptavidin possesses no carbohydrate chain (2) and has a lower isoelectric point, whereas avidin is a glycoprotein (8). Thus streptavidin is more useful for most applications than avidin because of its reduced nonspecific binding. In addition, streptavidin has no sulfur-containing amino acids (9), whereas avidin contains a disulfide-linkage as well as two methionine residues per subunit (3, 8). This also offers some advantages for a variety of applications.

We are interested in constructing expression vectors to produce streptavidin-containing fusion proteins. Such proteins could be detected and purified easily by their biotin binding. We are also interested in modifying the streptavidin gene to produce mutants that would expand the applications of the streptavidin–biotin system. As the first step in such studies, we attempted to express the cloned streptavidin gene from *S. avidinii* (9) in *Escherichia coli*. Although the streptavidin gene is extremely lethal to the host cells, it can be expressed efficiently by using T7 RNA polymerase/T7 promoter expression systems (10, 11). Here we describe several expression vectors and expression systems for the cloned streptavidin gene and also show some properties of the expressed streptavidin.

**MATERIALS AND METHODS**

**Bacteria, Bacteriophages, and Lysogens.** *E. coli* strains HMS174 (F⁻ hsdR recA Rif²) (12) and BL21 (F⁻ ompT hsdS gal) (10, 13) were used for cloning and expression. *E. coli* strain ED8739 (F⁻ metB hsdS supE supF) (10) was used as the host for growing CE6. The bacteriophage λ derivative CE6 (10) was used for delivering T7 RNA polymerase. CE6 carries the T7 RNA polymerase gene (T7 gene 1) (14) under the p₁ and p₂ promoters. BL21(DE3) and HMS174(DE3) are lysogens with the λ derivative DE3 integrated into the chromosome (10). DE3 carries the T7 RNA polymerase gene under the lacUV5 promoter in the int gene. These strains were gifts from F. W. Studier (Brookhaven National Laboratory).

**Plasmids.** pUC-SA1 (a gift from M. Uhlen and R. Johansson Royal Institute of Technology, Sweden) has the Fsp I–Sma I fragment (580 base pairs (bp)) of the streptavidin gene (9) inserted in the Sma I site of pUC8, in the same orientation as the lacZ gene. pT7-7 (a gift from S. Tabor, Harvard Medical School) is a derivative of pT7-5 (15) and has the Φ10 promoter (one of six strong T7 promoters that is located upstream of the T7 gene 10) (14) and the translation initiation site for the T7 gene 10 protein (bp 22,857–22,972 of T7 DNA) (14) inserted into bp 2065–4360 of pBR322 (16). This plasmid also has the polylinker region of pUC12 downstream of the initiation codon. pET-3a (17) (a gift from F. W. Studier) derived from pBR322 carries the Φ10 promoter and the first 11 codons of T7 gene 10 protein (bp 22,880–22,998 of T7 DNA) (14) followed by a BamHI linker. In addition, the transcription terminator Ψ (bp 24,106–24,228 of T7 DNA) (14) is placed downstream of the BamHI linker. pLysS and pLysE (11) (gifts from F. W. Studier) carry the T7 lysozyme gene (T7 gene 3.5, bp 10,665–11,296 of T7 DNA) (14) in pACYC184 (18). T7 lysozyme binds T7 RNA polymerase and inhibits its activity (19). Therefore, the presence of T7 lysozyme in the cell reduces the basal level of T7 RNA polymerase activity in the uninduced state (11). pLysE has the T7 lysozyme gene under the tet promoter of pACYC184, whereas pLysS has the gene in the opposite orientation, and thus the T7 lysozyme level with pLysE should be much lower than with pLysS (11).

**Construction of Expression Vectors.** Construction of the expression vectors was carried out using standard techniques (20). Restriction endonucleases, T4 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs or Boehringer Mannheim. pUC-SA1 was digested with Bgl I and treated with T4 DNA polymerase to generate blunt ends. The plasmid was then digested with BamHI, and the DNA fragments were electrophoresed on agarose gels. A 480-bp DNA fragment, which had the coding region for the 15th amino acid to the C terminus of mature streptavidin, was extracted and purified from the agarose using a DEAE-membrane (Schleicher & Schuell). The purified DNA fragment was ligated between the *Sma* I and BamHI sites of pT7-7 (2.46 kilobases (kb)) with T4 DNA ligase. The resulting plasmid is referred to as pTSA-1 (2.95 kb; Fig. 1).

pTSA-1 was digested with Nde I and BamHI, and a DNA fragment of 500 bp was purified using a DEAE-membrane after agarose gel electrophoresis. The purified DNA fragment was ligated between the Nde I and BamHI sites of pET-3a.
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in 10 ml of 2 mM EDTA/30 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100. Lysis occurred under these conditions because of the presence of T7 lysozyme in the cells. The cell lysate was stored at -70°C until used.

The frozen cell lysate stock was thawed at room temperature (≈22°C). MgSO₄ (1.0 M), DNase I (1 mg/ml, Sigma), and RNase A (1 mg/ml, Sigma) were added to final concentrations of 12 mM, 10 μg/ml, and 10 μg/ml, respectively, and the mixture was allowed to stand for 15 min at room temperature to reduce the viscosity of the lysate. The cell lysate was centrifuged at 39,000 × g for 15 min, and the precipitate was washed again with 10 ml of 2 mM EDTA/30 mM Tris-HCl, pH 8.0/0.1% Triton X-100 followed by centrifugation as above. The precipitate was dissolved in 5 ml of 6 M guanidine hydrochloride (pH 1.5) with gentle stirring and dialyzed against the same solution. The dialysate was then dialyzed against 0.2 M NaHCO₃ (pH 8.7) to remove guanidine hydrochloride. The dialysate was centrifuged at 39,000 × g for 15 min and the supernatant was used as the expressed streptavidin fraction.

**Protein Characterization.** Gel-filtration chromatography was conducted at room temperature using a Sephacryl S-200 HR column (Pharmacia). Detailed conditions are given in the legend to Fig. 6. The biotin-binding ability of streptavidin was determined by the bentonite adsorption method (22) using D-[carbonyl-14C]biotin (52 mCi/mmol; 1 Ci = 37 GBq; Amersham). SDS/PAGE analyses were performed with a discontinuous buffer system (23) using a 15% polyacrylamide gel. The proteins were stained with 0.15% Coomassie brilliant blue (Sigma) dissolved in 45% (vol/vol) methanol/10% (vol/vol) acetic acid. Protein concentration was determined by the microbiuret method (24) using bovine serum albumin as the standard or by using ε₂₈₀ = 3.4 for streptavidin (25).

**RESULTS AND DISCUSSION**

Expression by Infection with Bacteriophage CE6. The efficacy of the streptavidin–biotin detection system originates from the high and specific binding affinity of streptavidin for biotin. However, this characteristic is a severe disadvantage in attempts to express the cloned streptavidin gene. When the streptavidin gene is expressed, even in small amounts, the gene product binds biotin molecules, which are essential for cell metabolism; this causes the death of the host cell.

We first constructed the expression vector pT7-SAIH, which contained the entire streptavidin gene including the signal peptide and also encoded 25 vector-derived amino acids. pT7-SAIH could transform HMS174(DE3), a lysogen carrying the T7 RNA polymerase gene, and a protein of Mr 22,400, consistent with the deduced amino acid sequence, was expressed efficiently after induction of the T7 RNA polymerase gene (data not shown). However, the expressed protein did not show any biotin-binding ability, suggesting that the signal peptide region or the 25 vector-derived amino acids inactivated the protein. These results encouraged us to construct expression vectors for the streptavidin gene without the signal peptide—i.e., mature streptavidin.
We then constructed the expression vector pTSA-1, in which the coding region for the signal peptide was omitted. In addition, the coding sequence corresponding to several amino acids in the N-terminal region of the mature streptavidin is truncated (Fig. 1B). Streptavidin preparations purified from the culture medium of *S. avidini* are usually proteolyzed at both the N and the C termini to produce a minimal size molecule (9, 26-28), called core streptavidin. The N terminus encoded by pTSA-1 has almost the same structure as that of the core streptavidin (26, 28) preceded by five additional vector-derived amino acids (Fig. 1B).

pTSA-1 could transform HMS174 and could be stably maintained in the cell during growth. We tried to express the streptavidin gene by infection with bacteriophage CE6, which delivers T7 RNA polymerase to the cell. The SDS/PAGE patterns of total cell protein (Fig. 2A) show that, with increasing incubation time after infection, a protein band at Mr 16,000 increases in intensity. This band could not be observed at the time of infection. The molecular weight of this protein is consistent with that of the streptavidin subunit encoded in pTSA-1. In Western blots (Fig. 2B), only one band with Mr 16,000 cross-reacted with anti-streptavidin. Thus the Mr 16,000 protein was the product of the cloned streptavidin gene.

HMS174 transformed with pTSA-1 grew well in enriched medium, such as LB, but grew poorly in M9 minimal medium. Western blots (Fig. 2B) show that a small amount of streptavidin was expressed even before infection (not apparent in Fig. 2). This suggests that the target gene could be transcribed by *E. coli* RNA polymerase even if placed under the T7 promoter. When expressed streptavidin binds biotin in the cell, additional biotin molecules can apparently be taken up from the medium. This idea is supported by the fact that the cells can grow well in M9 medium supplemented with 8.2 μM biotin. Biotin supplementation is presumably indispensable for the host cells to maintain stably the plasmid carrying the streptavidin gene.

The above method, where T7 RNA polymerase is delivered by infection with bacteriophage CE6, successfully expresses the cloned streptavidin gene in *E. coli*. This system is applicable to many toxic genes that cannot be maintained in cells carrying the T7 RNA polymerase gene (10, 11). However, the method is inconvenient for routine expression experiments, because purified bacteriophage stock must be prepared each time. In addition, the multiplicity of infection and the growth conditions affect the expression efficiency of the target gene considerably (10, 11). The expression effi-

ciency of the cloned streptavidin gene varied with every experiment. Thus we attempted, instead, to express the cloned streptavidin gene using lysogen systems.

Expression Using pTSA-1 in Lysogen Systems. pTSA-1 could transform HMS174(DE3), a lysogen carrying the T7 RNA polymerase gene; however, the plasmid was unstable and lost during growth. In addition, this plasmid could not transform BL21(DE3), a lysogen containing the T7 RNA polymerase gene, even in the presence of pLysS or pLysE carrying the T7 lysozyme gene. In contrast, HMS174(DE3) (pLysS) or HMS174(DE3)(pLysE), lysogens carrying the T7 RNA polymerase gene and transformed with pLysS or pLysE, respectively, could be transformed with pTSA-1 and maintained the plasmid stably during the growth, suggesting that streptavidin is one of a small fraction of target genes that are more stable in HMS174(DE3) derivatives than in the equivalent derivatives of BL21(DE3) (11). Therefore, the expression of the streptavidin gene using the latter systems was characterized.

SDS/PAGE analysis of total cell protein (Fig. 3A) shows that a protein at Mr 16,000 was expressed extensively in cells carrying pTSA-1, but this protein was not observable in cells without pTSA-1. At the time of induction, little trace of this band could be seen. However, the cellular content of this protein increased with incubation time after the induction. In Western blots (Fig. 3B), only the Mr 16,000 protein cross-reacted with anti-streptavidin in the cells with pTSA-1. These results show clearly that the streptavidin gene was expressed efficiently by using this lysogen system. The expression efficiency was higher for HMS174(DE3)(pLysS)(pTSA-1) than for HMS174(DE3)(pLysE)(pTSA-1). The expressed streptavidin content in HMS174(DE3)(pLysS)(pTSA-1) was estimated to be >25% of the total cell protein 3 hr after induction.

![Fig. 2. Expression of streptavidin by infection with bacteriophage CE6.](image)

![Fig. 3. Expression of streptavidin using pTSA-1 in lysogen systems.](image)
Expression Using pTSA-2 in Lysogen Systems. Transcription by T7 RNA polymerase is very active (14), yielding long RNA molecules. If RNA synthesis were terminated just after the coding region, production of coding region RNA should become more efficient. This would increase the expression of the target gene. To test this, we constructed the expression vector pTSA-2, which has the T7 transcription terminator 40 bp downstream from the coding region. Surprisingly, pTSA-2 could transform both HMS174(DE3) and BL21(DE3) in the presence of pLysS or pLysE, whereas pTSA-1 could not transform BL21(DE3) even in the presence of pLysS or pLysE. The \( \Phi 10 \) promoter-containing fragment in pTSA-2 lacks bp 22,857–22,879, which have significant promoter activity for E. coli RNA polymerase (17), whereas the fragment in pTSA-1 retains this sequence. Therefore, smaller amounts of streptavidin should be expressed from pTSA-2 than from pTSA-1 in the uninduced state, resulting in a lower toxicity to the host cell.

BL21(DE3)(pLysS) could not maintain pTSA-2 stably. Cells that lost the target plasmid over-grew the culture during the incubation after the induction of the T7 RNA polymerase gene, and thus the expression of the \( M_s \), 16,000 protein was poor (Fig. 4A). However, BL21(DE3)(pLysE) could maintain pTSA-2 stably, and the \( M_s \), 16,000 protein was expressed efficiently (Fig. 4A). Western blots (Fig. 4C) show that only the \( M_s \), 16,000 protein cross-reacted with anti-streptavidin in the cells with pTSA-2, indicating that the \( M_s \), 16,000 protein was the product of the streptavidin gene. For BL21(DE3)-(pLysE)(pTSA-2), the expressed streptavidin accounted for >35% of the total cell protein 5 hr after the induction.

In contrast, both HMS174(DE3)(pLysS)(pTSA-2) and HMS174(DE3)(pLysE)(pTSA-2) could express the \( M_s \), 16,000 protein after induction (Fig. 4B). In Western blots (Fig. 4C), only the \( M_s \), 16,000 protein cross-reacted with anti-streptavidin. The expression efficiency was higher for HMS174(DE3)(pLysS), consistent with what was observed with expression using pTSA-1 (Fig. 3). The expression efficiency of HMS174(DE3)(pLysS)(pTSA-2) 3 hr after the induction seems almost the same as that of BL21(DE3)(pLysE)(pTSA-2) 5 hr after the induction. A slightly higher expression efficiency could be found in the cells transformed with pTSA-2 than those with pTSA-1 (Fig. 3) in this system.

We conclude that the cloned streptavidin gene is expressed efficiently in E. coli by using the lysogen systems with the addition of T7 lysozyme to reduce the basal level of T7 RNA polymerase activity in the uninduced state.

**Purification and Properties of Expressed Streptavidin.** BL21 has a potential advantage as an expression system. This cell should lack the lon protease and the ompT protease (10, 13). Because these proteinases may cause degradation of expressed proteins in the cell and during purification steps (13), we used BL21(DE3)(pLysE)(pTSA-2) incubated for 5 hr after induction as the source for purification attempts.

Most of the expressed streptavidin was insoluble in the cell, probably forming an inclusion body as generally observed in E. coli overexpression systems. However, when the insoluble protein fraction was dissolved by dialyzing against 6 M guanidine hydrochloride (pH 1.5) and then guanidine hydrochloride was removed by dialysis, the expressed streptavidin became soluble. The resulting supernatant was composed almost solely of the expressed streptavidin (Fig. 5), demonstrating that the expressed streptavidin could be purified almost to homogeneity by this simple procedure. Nucleic acid contamination was sometimes found, but this could be removed easily by affinity chromatography using 2-iminobiotin as the ligand (29, 30) or by gel-filtration chromatography. The yield of the expressed streptavidin ranged from 3.9 to 6.5 mg per 100 ml of culture. This yield was almost the same as that from S. avidinii, which takes 5–10 days to culture and requires chromatographic purification steps (29–31).

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**Fig. 4.** Expression of streptavidin using pTSA-2 in lysogen systems. Total cell protein of BL21(DE3)(pLysS), BL21(DE3)-(pLysE), HMS174(DE3)(pLysS), and HMS174(DE3)(pLysE) with or without pTSA2 was subjected to SDS/PAGE. (A and B) Stained protein. (C) Western blots. In lanes b–e and g–j, the number above each lane is the time after the induction in hr. Lanes: a, core streptavidin; b, BL21(DE3)(pLysS); c, BL21(DE3)(pLysS)(pTSA-2); d, BL21(DE3)(pLysE); e, BL21(DE3)(pLysEpTSA-2); f, Left, standard proteins; f, Right, prestained standard proteins; g, HMS174(DE3)(pLysS); h, HMS174(DE3)(pLysS)(pTSA-2); i, HMS174(DE3)(pLysE); j, HMS174(DE3)(pLysEpTSA-2). For A and B, each lane contains the total cell protein from 208 ml of culture except for lane b at 3 hr, lane d at 3 hr, lane g at 3 hr, lane h at 3 hr, and lane i at 3 hr that contain 125 ml, and for lane d at 5 hr and lane i at 5 hr that contain 83 ml. For C, the cell protein from 42 ml of culture was applied to each lane.

**Fig. 5.** SDS/PAGE analysis of the total protein of each fraction during purification of the expressed streptavidin using BL21(DE3)-(pLysEpTSA-2). Lanes: a, core streptavidin; b, at the time of induction; c, at 5 hr after the induction; d, soluble fraction after the lysis; e, soluble fraction after solubilization of the insoluble proteins by dialysis against 6 M guanidine hydrochloride (pH 1.5) followed by dialysis against 0.2 M NaHCO3 (pH 8.7); f, insoluble fraction after the solubilization in guanidine hydrochloride followed by the removal of guanidine hydrochloride; g, molecular weight standard proteins.
The purified streptavidin bound 3.5–3.9 molecules of biotin per tetramer of the subunit, indicating that the protein has almost full-biotin binding ability (4 molecules of biotin per tetramer). When dialysis against 6 M guanidine hydrochloride (pH 1.5) was eliminated from the purification procedures, the biotin-binding ability was reduced by 20%, which reveals that some of the expressed streptavidin had bound biotin in the cell or during purification. Although solubilization in 6 M guanidine hydrochloride at neutral pH is sufficient to isolate the expressed streptavidin, dialysis against 6 M guanidine hydrochloride (pH 1.5) is indispensable for a more active streptavidin preparation.

The purified streptavidin showed a sharp peak at Mr 64,000 (tetramer of the subunit) with a broad shoulder at higher molecular weight range (Fig. 6) on gel-filtration chromatography. On SDS/PAGE analysis, only one band could be observed at Mr 16,000 even for the fractions at the higher molecular weight range (data not shown). These results indicate that some of the expressed streptavidin aggregated into oligomers, which accounted for 30–50% of the total protein. Earlier reports suggested that the full-length gene product tends to aggregate (26, 28, 30) and both termini of the molecule are disordered or relatively flexible (27, 32). Because the N-terminal region has been truncated in our streptavidin preparation by the deletion of the corresponding coding region, it is likely that the C-terminal region of the mature streptavidin is responsible for the aggregation, although participation of the N-terminal region cannot be excluded. Because both terminal regions are rich in hydrophilic amino acid residues (13), ionic or hydrogen bonds might be responsible for the intermolecular interactions. With an efficient expression system now in hand, it should be possible to explore this issue further along with many other outstanding issues relevant to streptavidin’s unusual properties and great usefulness in numerous biotechnology applications.

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