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Constitutive production and efficient secretion of soluble full-length streptavidin by an *Escherichia coli* 'leaky mutant'

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ABSTRACT

Due to its various applications the protein streptavidin is a highly interesting target for heterologous production. This study focuses on different *Escherichia coli*-based constructs targeting a high-level expression and secretion of streptavidin to the medium. The effect of various promoters, variants of the target gene, leader sequences and host strains on expression and secretion into the culture broth was analyzed. Constitutive production of full-length streptavidin fused with the leader sequence of the *bglA* gene from *Bacillus amyloliquefaciens* by the periplasmic 'leaky mutant' *E. coli* JW1667-5 (*Δlpp-752:kan*) at 30 °C generated the highest yield of the conditions tested, surpassing the extracellular concentration of a conventional T7-based expression system. Supplementation of the medium by the non-ionic surfactants Triton® X-100 and X-45 led to an improved secretion of the protein to the culture supernatant. Tetrameric concentrations of streptavidin of 2790 ± 166 nM were reached in shake flasks at a productivity of 49.6 nM h^{-1}. Optimization of conditions led to a successful transfer to the bioreactor, yielding a maximal concentration of 2608 ± 169 nM and a productivity of 65.2 nM h^{-1} in fed-batch operation. The proportion of biotin-blocked binding sites of 8.3 ± 4.3% indicated a highly bioactive product.

1. Introduction

The homotetrameric protein streptavidin (SAV) binds one molecule of the vitamin biotin per monomer characterized by a dissociation constant K_d of 4×10^{-14} M (Green, 1990) to 10^{-15} M (Bayer and Wilchek, 1990). Being one of the strongest noncovalent biological interactions the biotin-binding ability of SAV can be used in a large variety of applications. Among them are the recovery of biotinylated (Rösli et al., 2008) or peptide-tagged (Skerra and Schmidt, 1999) proteins, the localization and detection of various proteins (Zwart and Lewis, 2008), and strategies for drug targeting (Muzykantov et al., 1999). Due to its biotin-binding properties SAV is known to be toxic to bacteria (Chaiet and Wolf, 1964). This antibiotic effect probably is the reason for its natural occurrence but complicates its heterologous production using *Escherichia coli*, especially for strategies based on intracellular expression.

In contrast to avidin from chicken egg white SAV has no sulfur-containing amino acids (Argaraña et al., 1986), a lower isoelectric point (Chaiet and Wolf, 1964), and is not glycosylated which offers advantages concerning applications of SAV due to less nonspecific interactions with substances other than biotin (Bayer and Wilchek, 1990).

The native SAV gene codes for 159 amino acids (16.5 kDa per monomer). However, various N- and C-terminally shortened species naturally occur due to limited proteolysis (Bayer et al., 1989; Sano et al., 1995). The so-called 'core streptavidins', smallest forms of SAV still maintaining the biotin-binding properties of full-length SAV, consist of 118-127 amino acids and show increased solubility (Pähler et al., 1987; Sano et al., 1995). Lacking the nonfunctional termini, core SAV may exhibit advantageous properties in production by reducing the metabolic burden for the host as well as for applications due to an improved accessibility of the binding site for biotinylated macromolecules.

Various expression systems for the heterologous production of SAV are described in literature. Most of them are based on the biotechnological model bacterium *E. coli* (Sano and Cantor, 1990; Miksch et al., 2008; Gallizia et al., 1998; Wu and Wong, 2006; Chen et al., 2014). These systems usually lead to the intracellular accumulation of the protein and inclusion body formation. Others report soluble intracellular SAV partly overcoming its toxicity. Only Miksch et al. (2008) achieved extracellular accumulation of the protein in its active form. In regard to the necessity of cell disruption, additional purification steps and possibly renaturation for

Abbreviations: SAV, streptavidin; vvm, volume per volume and minute.

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intracellular products the secretion of bioactive SAV to the medium is desirable. Other studies focus on production of SAV by *Pichia pastoris* (Casteluber et al., 2012; Nogueira et al., 2014) and *Bacillus subtilis* (Wu et al., 2002; Wu and Wong, 2002). Homologous production has been reported using the natural producers *Streptomyces avidinii* (Kolomiets and Zdor, 1998; Müller et al., 2013) and *Streptomyces lavendulae*.

Using inducible expression systems the metabolic burden is reduced during bacterial growth, which commonly leads to higher growth rates compared to constitutive systems. This difference is even more pronounced if the target protein is toxic (Baneyx, 1999). Approaches based on the inducible T7 expression system are well-established and frequently used for heterologous protein production by *E. coli*. A typical, commercial system consists of *E. coli* KRX, a strain characterized by low basal expression (Hartnett et al., 2006), and plasmids from the pET-series (Novagen, Madison, Wisconsin, USA). Uncoupling of growth and production can also be achieved by the use of stationary phase promoters.

However, for the production of SAV Miksch et al. (2008) obtained best results using a constitutive promoter (P_{bglA}). Secretion to the culture broth was achieved by the *phoA* signal sequence for periplasmic localization of the target protein and a gene coding for a bacteriocin release protein (BRP) driven by the stationary phase promoter P_{stat52} (Miksch et al., 2005) in order to permeabilize the outer membrane in the stationary growth phase. This active disruption of the outer membrane may be avoided by the use of periplasmic 'leaky mutants'. The K12-derived *E. coli* strain JW1667-5 (*Δlpp-752:kan*) was created by a single knockout of the murein lipoprotein gene *lpp*, affecting the integrity of its cell envelope (Baba et al., 2006; DiRienzo et al., 1978). For similar strains lacking the *lpp* gene, Ni et al. (2007) describe enhanced permeability of the outer membrane, but no significant change in cell growth, carbon metabolism or fatty acid composition compared to the wild type. The strain of Ni et al. (2007) has already been used for the production and secretion of a maltose binding protein, a xylanase and a cellulase (Shin and Chen, 2008). Thus, the system seems promising for secreting small proteins like SAV to the cultivation medium.

Transport of target proteins to the periplasm has several advantages compared to intracellular localization, e.g., reduced protease activity (Choi and Lee, 2004). In *E. coli*, this can be achieved by using signal peptides for the general secretory pathway (Sec). In the case of toxic proteins like SAV this pathway is advantageous because the protein remains (partially) unfolded during transport (Pugsley, 1993), reducing the concentration of bioactive intracellular product. However, secretion efficiency strongly depends on target protein, signal sequence, and host strain (Choi and Lee, 2004). For the secretion of SAV Miksch et al. (2008) used the signal sequences of the *phoA* gene for the alkaline phosphatase from *E. coli* and of the *bglA* gene for the β-glucanase from *Bacillus amyloliquefaciens*. The application of the *phoA* signal sequence led to secretion efficiencies of almost 100%.

1.1. Performance of selected expression systems

Müller et al. (2013) used the natural producer *S. avidinii* for production, yielding 39.2 μM of extracellular, tetrameric SAV in 14 days (114 nM h^{-1}) in a fed-batch process. However, due to the complex morphology and sensitivity of the organism and long cultivation times an efficient heterologous expression system for the SAV gene is desirable.

Miksch et al. (2008) reported a maximal concentration of 1.6 μM of extracellular SAV in 8 h (200 nM h^{-1}) using *E. coli* in a stirred tank bioreactor. Recently, heterologous expression systems were published based on the yeast *P. pastoris* and complex methanol-based fermentation processes (Nogueira et al., 2014; Casteluber et al., 2012). Nogueira et al. (2014) reached a final extracellular SAV concentration of 11 μM in 164 h (67 nM h^{-1}) in the bioreactor. Casteluber et al. reported even higher concentrations in the range of 71 μM in spinner flasks.[1]

1.2. Goals of this study

The system of Miksch et al. (2008) as mentioned above led to remarkable productivities in short cultivation periods. However, maximal biomass concentrations were low (<10 g L^{-1}, OD_{600} < 25). Furthermore, the system contained an Ω-Cm transposon (Miksch et al., 1997) to avoid uncontrolled expression of the bacteriocin release protein-encoding gene, causing serious problems in the genetic stability of the producer strains. To achieve higher dry cell weight (DCW) concentrations and a more stable expression over longer periods of time, this study focused on an a novel secretion strategy avoiding an active disruption of the outer membrane of *E. coli* based on the application of the periplasmic 'leaky mutant' JW1667-5. Comparable deletion mutants have not been used for the production of SAV before.

On the genetic level, several promoters, leader peptides, and a variety of SAV genes differing in chain length and codon usage were applied. One of the research questions in this context was if shortened genes (core SAV) can be used for SAV production by *E. coli*. Based on an optimized genetic configuration, the performance of the new construct was compared to a conventional producer strain (*E. coli* KRX).

So far, none of the studies performed targeting expression of the SAV gene by *E. coli* contained detailed investigations on the optimization of process conditions. Corresponding experiments where therefore integrated in the work plan applying various culture conditions and cultivation parameters (medium, temperature, supplements) in batch and fed-batch operation. Thus, factors on all major levels of the development of recombinant production processes were part of this study to get a more holistic view of *E. coli*'s potential for the production and secretion of SAV.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strain *E. coli* KRX (Promega, Fitchburg, Wisconsin, USA) was used for cloning work and as a host for heterologous gene expression based on the T7 system. The K12 BW25113-derived strain *E. coli* JW1667-5, purchased from the Coli Genetic Stock Center (CGSC, Keio Knockout Collection, Yale University, New Haven, Connecticut, USA), was used as a second expression host. *S. avidinii* (CBS 730.72; DSM 40526) was ordered from CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands).

The plasmids constructed during this project are given in Table 1, which contains information on designations and features. The pET24a vector backbone was purchased from Novagen (Madison, Wisconsin, USA) and used in combination with *E. coli* KRX (plasmids 7–10). For plasmids 1–6 the pEL vector backbone was used, constructed and generously provided by Maurice Telaar of Bielefeld University (Germany). Both backbones are shown in Fig. S2 (Supplementary material).

2.2. Plasmid construction and verification of cloning steps

The plasmids used in this project were constructed as described by Gibson et al. (2009) using the isothermal reaction protocol (50 °C, 1 h) and a master mix purchased from NEB (Ipswich, Massachusetts,

[1] Assuming a molecular weight of the core SAV of 56 kDa per tetramer.