

OBJECTS OF THE INVENTION

Thus, the object of the present invention was to provide improved DNA constructs, as well as a bacterial host cell and method suitable for the efficient production of recombinant proteins, such as monoclonal antibodies or fragments thereof in a bacterial host cell in a high yield, particularly when such recombinant proteins are intended for therapeutic applications. The recombinant protein may be ranibizumab.

SUMMARY

In a first aspect, the present disclosure relates to a DNA construct for expressing ranibizumab in a bacterial host cell, wherein ranibizumab comprises (i) a light chain comprising the amino acid sequence of SEQ ID 3, and (ii) a heavy chain comprising the amino acid sequence of SEQ ID 4, wherein said DNA construct comprises a nucleotide sequence encoding ranibizumab, wherein said DNA construct further comprises at least one nucleotide sequence encoding a signal peptide which is operably linked in the direction of transcription to the nucleotide sequence encoding the light chain of ranibizumab and/or the heavy chain of ranibizumab, wherein said DNA construct further comprises nucleotide sequences encoding:

- *rhaBAD* promoter,
- RhaR transcription activator,
- RhaS transcription activator,
- an antibiotic resistance marker,
- a promoter operably linked to the nucleotide sequence encoding the antibiotic resistance marker
- *rrnB* T1 terminator
- *rrnB* T2 terminator, and
- a pMB1 origin of replication.

An example of the present disclosure relates to a DNA construct for expressing ranibizumab in a bacterial host cell, wherein ranibizumab comprises (i) a light chain comprising the amino acid sequence of SEQ ID 3, and (ii) a heavy chain comprising the amino acid sequence of SEQ ID 4, wherein said DNA construct comprises a nucleotide sequence encoding ranibizumab, wherein said DNA construct further comprises at least one nucleotide sequence encoding a signal peptide which is operably linked in the direction of transcription to the

nucleotide sequence encoding the light chain of ranibizumab and/or the heavy chain of ranibizumab, wherein said DNA construct comprises nucleotide sequences encoding:

- *rhaBAD* promoter,
- RhaR transcription activator,
- 5 - RhaS transcription activator,
- an antibiotic resistance marker,
- at least one terminator, and
- an pMB1 origin of replication.

10 In a preferred example, said DNA construct further comprises a nucleotide sequence encoding a promoter operably linked to the nucleotide sequence encoding the antibiotic resistance marker.

In a preferred example, the DNA construct further comprises nucleotide sequences encoding rrnB T1 terminator and rrnB T2 terminator.

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In a preferred example, the DNA construct is characterized by:

- the antibiotic resistance marker is a kanamycin resistance marker, preferably a kanamycin resistance marker comprising the nucleotide sequence of SEQ ID 12 or a sequence with at least 90 % sequence identity thereto;
- the promoter operably linked to the nucleotide sequence encoding the antibiotic resistance marker is an AmpR promoter, preferably an AmpR promoter comprising the nucleotide sequence of SEQ ID 13 or a sequence with at least 90 % sequence identity thereto;
- the *rrnB* T1 terminator comprising the nucleotide sequence of SEQ ID 14 or a sequence with at least 90 % sequence identity thereto;
- the *rrnB* T2 terminator comprising the nucleotide sequence of SEQ ID 15 or a sequence with at least 90 % sequence identity thereto; and/or
- the pMB1 origin of replication comprising the nucleotide sequence of SEQ ID 16 or a sequence with at least 90 % sequence identity thereto.

In a preferred example, the DNA construct is characterized by:

- the antibiotic resistance marker being a kanamycin resistance marker comprising the nucleotide sequence of SEQ ID 12;
- the promoter operably linked to the nucleotide sequence encoding the antibiotic resistance marker is an AmpR promoter comprising the nucleotide sequence of SEQ ID 13;
- the *rrnB* T1 terminator comprising the nucleotide sequence of SEQ ID 14;
- the *rrnB* T2 terminator comprising the nucleotide sequence of SEQ ID 15; and/or
- the pMB1 origin of replication comprising the nucleotide sequence of SEQ ID 16.

In a preferred example, the DNA construct is characterized by:

- the *rhaBAD* promoter comprising the nucleotide sequence of SEQ ID 8 or a sequence with at least 90 % sequence identity thereto;
- the RhaR transcription activator comprising the nucleotide sequence of SEQ ID 9 or a sequence with at least 90 % sequence identity thereto;
- the RhaS transcription activator comprising the nucleotide sequence of SEQ ID 11 or a sequence with at least 90 % sequence identity thereto;
- the antibiotic resistance marker is a kanamycin resistance marker comprising the nucleotide sequence of SEQ ID 12 or a sequence with at least 90 % sequence identity thereto;

- the AmpR promoter comprising the nucleotide sequence of SEQ ID 13 or a sequence with at least 90 % sequence identity thereto;
- the *rrnB* T1 terminator comprising the nucleotide sequence of SEQ ID 14 or a sequence with at least 90 % sequence identity thereto;
- 5 - the *rrnB* T2 terminator comprising the nucleotide sequence of SEQ ID 15 or a sequence with at least 90 % sequence identity thereto; and
- the pMB1 origin of replication comprising the nucleotide sequence of SEQ ID 16 or a sequence with at least 90 % sequence identity thereto.

In a preferred example, the DNA construct is characterized by:

- 10 - the *rhaBAD* promoter comprising the nucleotide sequence of SEQ ID 8;
- the RhaR transcription activator comprising the nucleotide sequence of SEQ ID 9;
- the RhaS transcription activator comprising the nucleotide sequence of SEQ ID 11;
- 15 - the antibiotic resistance marker is a kanamycin resistance marker comprising the nucleotide sequence of SEQ ID 12;
- the AmpR promoter comprising the nucleotide sequence of SEQ ID 13;
- the *rrnB* T1 terminator comprising the nucleotide sequence of SEQ ID 14;
- the *rrnB* T2 terminator comprising the nucleotide sequence of SEQ ID 15; and
- 20 - the pMB1 origin of replication comprising the nucleotide sequence of SEQ ID 16.

In a preferred example, the DNA construct comprises a multiple cloning site comprising 17 restriction sites cleavable by restriction enzymes EcoRI, NdeI, NotI, XhoI, PspXI, PaeR71, BbsI, StyI, AvrII, BanI, Acc65I, KpnI, Eco53kI, SacI, BamHI, XbaI, SalI, AccI, PstI, SbfI, SphI and HindIII.

In a preferred example, the DNA construct comprises the nucleotide sequence of SEQ ID 1, or a sequence with at least 90 % sequence identity thereto, preferably the nucleotide sequence of SEQ ID 1.

In a preferred example, the nucleotide sequence encoding ranibizumab is operably linked to the *rhaBAD* promoter.

In a preferred example, the nucleotide sequence encoding ranibizumab comprises (i) a nucleotide sequence encoding for the light chain of ranibizumab comprising the sequence of

SEQ ID 5 or a sequence with at least 90 % sequence identity thereto, and/or (ii) a nucleotide sequence encoding for the heavy chain of ranibizumab comprising the sequence of SEQ ID 6 or a sequence with at least 90 % sequence identity thereto. In a further preferred example, said nucleotide sequence encoding ranibizumab comprises (i) a nucleotide sequence encoding for
5 the light chain of ranibizumab comprising the sequence of SEQ ID 5, and/or (ii) a nucleotide sequence encoding for the heavy chain of ranibizumab comprising the sequence of SEQ ID 6.

In an example, the nucleotide sequence encoding the signal peptide is operably linked in the direction of transcription to either one or both of the nucleotide sequence of SEQ ID 5 and SEQ ID 6.

10 In an example, the signal peptide is PelB (pectate lyase B). In a preferred example, the nucleotide sequence encoding the PelB signal peptide comprises a sequence of SEQ ID 7 or a sequence with at least 90 % sequence identity thereto; more preferably the nucleotide sequence encoding the PelB signal peptide comprises the sequence of SEQ ID 7.

In one example, the resulting PelB signal peptide comprises an amino acid sequence of SEQ
15 ID 18 [6]: MKYLLPTAAAGLLLLAAQPAMA.

In a preferred example, the DNA construct comprises the sequence of SEQ ID 17 or a sequence with at least 90 % sequence identity thereto, preferably comprises the sequence of SEQ ID 17.

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A second aspect of the disclosure relates to an expression vector comprising any of the DNA constructs as described herein.

A third aspect of the disclosure relates to a bacterial host cell comprising a DNA construct as
25 described herein or the expression vector as described herein, wherein said bacterial host cell is preferably an *Escherichia coli* cell, more preferably an *E. coli* K-12 cell.

In a preferred example, said bacterial host cell either comprises (i) a chromosome which comprises a mutation in the nucleic acid sequence of the *rhaB* gene which renders RhaB inactive, or (ii) a chromosome in which the nucleotide sequence encoding RhaB is deleted.

5 In a preferred example, said bacterial host cell is an *Escherichia coli* W3110 cell, preferably comprising a chromosome which comprises a frame shift-mutation in the nucleotide sequence encoding RhaB.

10 In a preferred example, said bacterial host cell is an *Escherichia coli* W3110 cell comprising a chromosome which comprises nucleotide sequence of SEQ ID 2 or a sequence with at least 90 % sequence identity thereto, preferably the nucleotide sequence of SEQ ID 2, wherein optionally the chromosome of the bacterial host cell further comprises a nucleotide sequence encoding RhaT.

15 A fourth aspect of the disclosure relates to a method of producing ranibizumab comprising the step of exposing the bacterial host cell as described herein to rhamnose, thereby inducing expression of ranibizumab. In a preferred example, the method further comprises the step of recovering ranibizumab from the bacterial host cell; and optionally further comprises one or more step(s) of purifying the recovered ranibizumab, preferably by one or more chromatography steps.

20 Another example of the disclosure relates to a method of producing ranibizumab, comprising the steps of:

cloning nucleotide sequence encoding a recombinant protein into a DNA construct which comprises a nucleotide sequence of SEQ ID 1 or a sequence with at least 90 % sequence identity thereto, preferably a nucleotide sequence of SEQ ID 1, such that the nucleotide
25 sequence encoding ranibizumab protein is operably linked to the *rhaBAD* promoter; and

- a. introducing the resulting nucleotide sequence into a bacterial host cell comprising a chromosome which comprises a mutation or modification which disables rhamnose metabolism.

30 In a preferred example, the method further comprises the step of exposing the bacterial host cell to rhamnose, thereby inducing expression of the recombinant protein.

In another example, the method further comprises the step of recovering ranibizumab from the bacterial host cell; and optionally further comprises one or more step(s) of purifying the recovered ranibizumab, preferably by one or more chromatography steps.

In a preferred example, said bacterial host cell is an *Escherichia coli* cell, more preferably an *E. coli* K-12 cell, most preferably an *E. coli* W3110 cell.

In a preferred example, said bacterial host cell either comprises (i) a chromosome which comprises a mutation in the nucleotide sequence of the *rhaB* gene which renders RhaB inactive, or (ii) a chromosome in which the nucleotide sequence encoding RhaB is deleted.

In a preferred example, said bacterial host cell is an *E. coli* W3110 cell comprising a chromosome which comprises a frame shift-mutation in the nucleotide sequence encoding RhaB.

In a preferred example, said bacterial host cell is an *E. coli* W3110 cell comprising a chromosome which comprises the nucleotide sequence of SEQ ID 2 or a sequence with at least 90 % sequence identity thereto, preferably the nucleotide sequence of SEQ ID 2, wherein optionally the chromosome of the bacterial host cell further comprises a nucleotide sequence encoding RhaT.

In a preferred example, said nucleotide sequence encoding ranibizumab comprises (i) a nucleotide sequence encoding the light chain of ranibizumab comprising the sequence of SEQ ID 5 or a sequence with at least 90 % sequence identity thereto, and/or (ii) a nucleotide sequence encoding for the heavy chain of ranibizumab comprising the sequence of SEQ ID 6 or a sequence with at least 90 % sequence identity thereto; preferably said nucleotide sequence encoding the recombinant protein comprises (i) a nucleotide sequence encoding for the light chain of ranibizumab comprising the sequence of SEQ ID 5, and/or (ii) a nucleotide sequence encoding for the heavy chain of ranibizumab comprising the sequence of SEQ ID 6.

In a preferred example, a nucleotide sequence comprising SEQ ID 5 and/or SEQ ID 6 further comprises a nucleotide sequence encoding a signal peptide which is operably linked in the direction of transcription to the nucleotide sequence of SEQ ID 5 and/or SEQ ID 6.

In a preferred example, said nucleotide sequence encoding the signal peptide is a nucleotide sequence encoding a PelB signal peptide, preferably the nucleotide sequence encoding the PelB signal peptide comprises the sequence of SEQ ID 7 or a sequence with at least 90 % sequence identity thereto; preferably the nucleotide sequence encoding the PelB

signal peptide comprises the sequence of SEQ ID 7 and is fused to the nucleotide sequence encoding the light chain and/or heavy chain of ranibizumab.

In a preferred example, the resulting nucleotide sequence to be introduced into the bacterial host cell comprises the sequence of SEQ ID 17 or a sequence with at least 90 % sequence identity thereto, preferably comprises the sequence of SEQ ID 17.

Another example of the disclosure relates to a bacterial host cell being an *E. coli* W3110 cell and comprising a chromosome which comprises a frame shift-mutation in the nucleotide sequence encoding RhaB. In an example, said *E. coli* W3110 host cell comprises a chromosome which comprises the nucleotide sequence of SEQ ID 2 or a sequence with at least 90 % sequence identity thereto, preferably the nucleotide sequence of SEQ ID 2. In a further example the chromosome of the bacterial host cell comprises a nucleotide sequence encoding RhaT.

One or more of the above indicated SEQ IDs 1-18 of the various examples of the disclosure (and examples thereof) may be replaced by a sequence with at least 90 % sequence identity thereto. The term "sequence identity" as used herein is used with regard to amino acid or nucleotide sequences and the sequence identity is over the entire length of the specified sequence. A sequence may thus be at least 90 percent, at least 92 percent, at least 95 percent, at least 96 percent, at least 97 percent, at least 98 percent or at least 99 percent, identical in sequence to the amino acid or nucleotide sequence specified. Such sequences of the disclosure thus include single or multiple nucleotide or amino acid alterations (additions, substitutions, insertions or deletions) to the sequences of the disclosure. At the amino acid level preferred sequences with the above defined sequence identity contain up to 5, e.g. only 1, 2, 3, 4 or 5, preferably 1, 2 or 3, more preferably 1 or 2, altered amino acids in the sequences of the disclosure.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 - Plasmid map for KTXHIS

Figure 2 - The nucleotide sequence of the multiple cloning site (MCS) of KTXHIS

Figure 3 - Plasmid map of KTXHIS-PelbLC-PelbHC