Method of Producing a Recombinant Protein

The present invention relates to a recombinant signal sequence, and a fusion protein comprising the signal sequence and a recombinant protein. Methods of producing the recombinant protein using the recombinant signal sequence are described. In particular, the recombinant signal sequence provides a method for controlling the viscosity of the fermentation, and/or controlling basal (pre-induction) expression of the recombinant protein.

Background of the invention

Large-scale and cost-effective manufacture, recovery and purification of recombinant proteins are important challenges for the biotechnology industry.

Microbial host cells are widely used organisms for the production of recombinant proteins. Considerations during production include host cell growth and expression of recombinant protein, protein titre, protein location (e.g. intracellular, extracellular, periplasmic, etc.), and selective recovery and purification from the final location of the recombinant protein. Balancing and optimising these different factors is not straightforward.

The Gram-negative eubacterium *Escherichia coli* (*E. coli*) is a robust expression system for use in industrial bioprocesses due to its well-characterized genetics; its ability to accumulate biomass rapidly using inexpensive substrate; the ease of process scale-up and the large number of available host strains and expression vectors. *E. coli* is a common host for the production of therapeutic proteins do not require complex post-translational modifications such as glycosylation. For disulphide bond formation, expression can be directed into the oxidizing environment of the periplasm to facilitate correct folding. This can be achieved by the use of N-terminal signal sequences, which are recognized by components of the cell's secretion system to initiate their targeting and passage through dedicated transport systems.

Accordingly, there is a need for improved methods of producing, recovering and purifying recombinant proteins.

Summary of the invention

The present invention provides a recombinant signal sequence comprising (a) the sequence MGRISSGG (SEQ ID NO: 1) or a variant thereof that differs in sequence by 1, 2, or 3 amino acid substitutions, deletions or insertions, and (b) a heterologous signal sequence C-terminal to (a).

The invention also provides a recombinant fusion protein comprising (a) the signal sequence as defined; and (b) a heterologous recombinant protein C-terminal to (a).

The invention also provides a recombinant nucleic acid sequence which encodes the signal sequence or the fusion protein as defined.

The invention also provides a recombinant nucleic acid sequence which encodes a signal sequence comprising the sequence MGRISSGG (SEQ ID NO: 1), wherein the nucleic acid sequence has been codon optimised for expression in a host cell.

The invention also provides a recombinant expression vector comprising the nucleic acid sequence as defined.

The invention also provides a method for producing a recombinant protein in a host cell comprising:

(a) culturing the host cell under conditions that allow expression of the recombinant protein from the recombinant expression vector as defined present in the host cell; and

(b) recovering the recombinant protein.

The invention also provides a method for controlling the viscosity of a host cell broth that expresses a recombinant protein, comprising:

culturing the host cell under conditions that allow expression of the recombinant protein, wherein the host cell comprises a recombinant nucleic acid sequence which encodes (i) a recombinant signal sequence comprising the sequence MGRISSGG (SEQ ID NO: 1) or a variant thereof that differs in sequence by 1, 2, or 3 amino acid substitutions, deletions or insertions, and (ii) the recombinant protein.

The invention also provides a method for controlling basal expression of a recombinant protein expressed by a host cell broth, comprising: culturing the host cell under pre-induction conditions,

wherein the host cell comprises a recombinant nucleic acid sequence which encodes (i) a recombinant signal sequence comprising the sequence MGRISSGG (SEQ ID NO: 1) or a variant thereof that differs in sequence by 1, 2, or 3 amino acid substitutions, deletions or insertions, and (ii) the recombinant protein.

Description of the Figures

Figure 1. The effect of different N-terminal signal sequences on the expression of dAb from *E. coli* W3110 in 24 dwp cultures. Key: B- dAb located extracellularly (supernatant); C- dAb located intracellularly (whole cell lysate: periplasmic + cytosolic). B + C - total dAb, B/(B + C) - proportion released. Summation of B and C allows visual estimation of total dAb formed. Samples were taken 24 h post-induction, reduced and

bands visualised by Coomassie stained SDS-PAGE. 'Std' denotes dAb standard at 0.5 $mg.mL^{-1}$ concentration.

Figure 2. Post-induction fermentation profiles for high cell density *E. coli* W3110 fed-batch cultures. N-terminal signal sequence and basal (pre-induction) expression level: (Δ), *OmpASS*, 0.30 $g.L^{-1}$, (□) *YceISS*, 0.64 $g.L^{-1}$ or (◊), *IvySS*, 0.061 $g.L^{-1}$. All results are taken post-induction of dAb formation with IPTG. (A) Biomass was estimated by OD_{600nm}; (B) Extracellular soluble dsDNA concentration; dAb yield was determined (C) intracellularly (whole cell lysate), (D) extracellularly (supernatant), as (E) total dAb formed (intracellular + extracellular) and as (F) percentage of total dAb in the extracellular space. Broth viscosity, μ, is described in terms of a power law relationship, $\mu = K \gamma^{n-1}$ where γ is shear rate, (G) K is the flow consistency index, and (H) n is the flow behaviour index. Results were taken from replicated fermenters (*n* = 2-4) averaged and error bars added corresponding to one standard deviation.

Figure 3. Signal sequences amino acid sequence alignments and evaluation of their codon adaptation indices. (A) Amino acid sequence alignments for YceISS (SEQ ID NO: 35) and IvySS (SEQ ID NO: 34) are referenced against the OmpASS (SEQ ID NO: 33). (B) Adaptiveness of each codon position was calculated as a ratio of that codon frequency against the most abundant synonymous codon with reference to the *E. coli* codon usage table derived from the Kazusa *E. coli* codon usage database. Amino acid sequences in 'B' are aligned from the first methionine after the Ivy'SS cassette.

Figure 4. Post-induction fermentation profiles for high cell density *E. coli* W3110 fed-batch cultures. N-terminal signal sequence and basal (pre-induction) expression level: (Δ), *IvyTruncSS*, 0.90 $g.L^{-1}$, (□), *Ivy'OmpASS*, 0.086 $g.L^{-1}$, (◊), *IvyOpt1SS*, 0.16 $g.L^{-1}$ and (♦), *IvyOpt2SS*, 0.089 $g.L^{-1}$. See Figure 2 for details. Results were taken from single fermentation samples (m = 3).

Figure 5. (A) Overall productivity calculated as the mass of extracellular dAb, per litre of broth per total post-induction time (B) centrifugation performance of *E. coli* harvest cultures expressing a dAb with different N-terminal signal sequences; OmpASS (48h post-induction), YceISS (48h), IvyTruncSS (55h), Ivy'OmpA (153h) and *Ivy* codon optimised signal sequences *IvyOpt1* (48h) and *IvyOpt2* (55h). Cell broths were exposed to conditions of: (■), no shear; (□), high shear stress (maximum energy dissipation rate (ε) of 0.53×10^6 $W.kg^{-1}$ for 20 s), and then processed by USD centrifugation (V = 2.0 mL, t = 420 s, Σ = 0.164 m^2, (see text), $V/t\Sigma = 2.9 \times 10^{-8}$ $m.s^{-1}$). Results were taken from replicated fermenters (*n* = 3) for OmpASS and YceISS or single fermenter samples for remaining signal

sequences. Sample characterization for (B) was in triplicate (*m* = 3). Results shown as mean +/- SD.

Detailed Description

The present disclosure involves the realisation that the use of specific signal sequences offer advantages in terms of fermentation, recombinant protein expression, and protein recoverability.

The signal sequences have been investigated for periplasmic secretion of a recombinant protein. The signal sequences offer the advantage of a low viscosity broth, arising from a low basal expression. An eight amino acid sequence comprised in the signal sequence is identified as key to lead to this low basal expression. Codon optimisation of the signal sequences was successful in retaining low basal expression and hence low viscosity broths while increasing fermentation productivity of the recombinant protein. Hence, the combination of high productivity and practicable clarification by full-scale centrifugation is achieved.

The sequence "MGRISSGG" (SEQ ID NO: 1) can be used N-terminal (5') to any signal sequence. For example, "MGRISSGG" (SEQ ID NO: 1) may be used N-terminal to a heterologous signal sequence. A heterologous signal sequence may be one that is not the endogenous IVY signal sequence. For example, the heterologous signal sequence is a periplasmic signal sequence from *E. coli*. For example, the *E. coli* periplasmic signal sequence is OmpA, MalE, PelB, OmpT, or LamB. For example, "MGRISSGG" (SEQ ID NO: 1) may be N-terminal to MKKTAIAIAVALAGFATVAQA (SEQ ID NO: 33), or any of the signal sequences in Table 3, or any *E. coli* periplasmic signal sequence.

The recombinant signal sequence comprises (a) the sequence MGRISSGG (SEQ ID NO: 1) or a variant thereof that differs in sequence by 1, 2, or 3 amino acid substitutions, deletions or insertions, and (b) a heterologous signal sequence C-terminal to (a). The heterologous signal sequence may be immediately C-terminal to MGRISSGG (SEQ ID NO: 1). For example, the recombinant signal sequence may comprise the sequence MGRISSGG (SEQ ID NO: 1) immediately N-terminal to a heterologous *E. coli* periplasmic signal sequence. For example, the recombinant signal sequence may comprise the sequence MGRISSGG (SEQ ID NO: 1) immediately N-terminal to the heterologous signal sequence MKKTAIAIAVALAGFATVAQA (SEQ ID NO: 33), to form recombinant signal sequence the MGRISSGGMKKTAIAIAVALAGFATVAQA (SEQ ID NO: 55).

The sequence "MGRISSGG" (SEQ ID NO: 1) may be varied by 1, 2, or 3 amino acid substitutions, deletions or insertions. The sequence "MGRISSGG" (SEQ ID NO: 1) plus a heterologous signal sequence may be varied by 1 to 10 amino acid substitutions,

deletions or insertions. The variant may retain the activity of "MGRISSGG" (SEQ ID NO: 1).

The amino acid substitution may involve the replacement of at least one amino acid with an amino acid from the same class, as defined below in Table 1.

Table 1

Amino acid class	Amino acid residue
Hydrophobic	VILMFWCGAPY
Uncharged polar	NQST
Basic polar	RKH

The at least one amino acid substitution, deletion or insertion may preserve the primary structure of the sequence "MGRISSGG" (SEQ ID NO: 1) by maintaining a consensus sequence with: (i) one or more hydrophobic amino acid(s), (ii) one or more basic polar amino acid(s), (iii) one or more hydrophobic amino acid(s), and (iv) one or more uncharged polar amino acid(s), as defined in Table 1.

The activity of "MGRISSGG" (SEQ ID NO: 1) when N-terminal (5') to a signal sequence may be defined as the control of basal expression (pre-induction expression) of a recombinant protein. Basal expression occurs during the pre-induction exponential growth phase of the host cell. During this growth phase, the recombinant protein should not be expressed or minimised, and instead the metabolic burden of the cells is focussed on growth. However, many signal sequences allow for some basal expression of the recombinant protein. The activity of "MGRISSGG" (SEQ ID NO: 1) or a variant thereof may be to control recombinant protein basal expression to a level of 0.5g/L or less at the time of induction, i.e. a pre-induction expression level of the recombinant protein of 0.5g/L or less. Use of a signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) can result in a low pre-induction expression level of the recombinant protein. For example, the pre-induction expression level of the recombinant protein may be ≤0.5g/L, ≤0.4g/L, ≤0.3g/L, ≤0.2g/L, ≤0.1g/L, or ≤0.05g/L.

Optionally in addition, the activity of "MGRISSGG" (SEQ ID NO: 1) when N-terminal (5') to a signal sequence may be defined as the control of viscosity of a host cell broth that expresses a recombinant protein. The host cell broth is comprised of the host cells plus the supernatant comprising the culture media. The broth viscosity can change during fermentation at the timepoints: pre-induction, at the point of induction, and post-induction. Use of a signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) can result in a low viscosity host cell broth during fermentation. Viscosity may be measured using methods

common in the field. For example, viscosity may be measured using the flow consistency index (K) or the apparent viscosity (μ).

For example, the host cell broth has a viscosity equivalent to a flow consistency index of K 0.1 Ns^nm^{-2} or less. For example, the host cell broth has a viscosity of K 0.05 Ns^nm^{-2} or less, K 0.04 Ns^nm^{-2} or less, K 0.03 Ns^nm^{-2} or less, K 0.02 Ns^nm^{-2} or less, or K 0.01 Ns^nm^{-2} or less.

For example, "low" viscosity may be equivalent to a value of K of about 0.001 Ns^nm^{-2} to 0.01 Ns^nm^{-2}, "medium" viscosity may be equivalent to a value of K of about 0.01 Ns^nm^{-2} to 0.1 Ns^nm^{-2}, and "high" viscosity may be equivalent to a value of K of about more than 0.1 Ns^nm^{-2}.

Alternatively, for example, the host cell broth has an apparent viscosity μ (as defined at a shear rate of 1 s^{-1}) of 0.1 Nsm^{-2} or less. For example, the host cell broth has an apparent viscosity of μ 0.05 Nsm^{-2} or less, μ 0.04 Nsm^{-2} or less, μ 0.03 Nsm^{-2} or less, μ 0.02 Nsm^{-2} or less, or μ 0.01 Nsm^{-2} or less. The apparent viscosity will vary with applied shear rate, values may be computed from values given of n the flow behaviour index and the chosen shear rate.

In one embodiment, this is the broth viscosity at the point of induction.

The nucleic acid sequence that encodes the recombinant signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) may be codon optimised for expression in a host cell. Methods for codon optimisation (also known as codon adaptation) are known in the art. For example, the codon adaptation index (CAI) may be referenced against the codon usage for all genes native to a particular cell line, or for high expressing genes only. All *E. coli* genes may be referenced, or all *E. coli* K12 genes, or *E. coli* K12 class II (high expressing) genes may be referenced.

The nucleic acid sequence that encodes the signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) may have a CAI score of 0.40 or above, 0.45 or above, 0.50 or above, 0.55 or above, 0.60 or above, 0.65 or above, 0.70 or above, 0.75 or above, 0.80 or above, 0.85 or above, or 0.90 or above.

For example, the nucleic acid sequence that encodes the signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) may comprise the codon optimised sequence:

"atgggacgtatttcttcgggcggaatgatgttcaaagcaatcaccacagtggcagctctggttattgctactagcgctatggct" (SEQ ID NO: 49); or

"atgggtcgtatctcttccggcggtatgatgttcaaagcaatcactaccgttgcagctctggttattgcgacctctgctatggca" (SEQ ID NO: 50).

Optionally in addition, the activity of "MGRISSGG" (SEQ ID NO: 1) or a variant thereof or a codon optimised sequence, may be to control recombinant protein expression upon induction to a level of 5g/L or more. Use of a signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) can result in a high post-induction expression level of the recombinant protein. For example, the post-induction expression level of the recombinant protein may be ≥5g/L, ≥6g/L, ≥7g/L, ≥8g/L, ≥9g/L, or ≥10g/L. The expression level of the recombinant protein may be total produced, or extracellular protein.

Optionally in addition, the activity of "MGRISSGG" (SEQ ID NO: 1) or a variant thereof or a codon optimised sequence, may be to control recombinant protein productivity upon induction to a level of 0.1g/L/h or more. Use of a signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) can result in a high recombinant protein productivity upon induction. For example, the post-induction productivity of the recombinant protein may be ≥0.10g/L/h, ≥0.11g/L/h, ≥0.12g/L/h, ≥0.13g/L/h, ≥0.14g/L/h, or ≥0.15g/L/h. The productivity of the recombinant protein may be total produced, or extracellular protein. The time (hours) may be calculated from the point of induction.

Optionally in addition, the activity of "MGRISSGG" (SEQ ID NO: 1) or a variant thereof or a codon optimised sequence, may be to control the % solids remaining (as defined by measurement of optical density at 600 nm) after centrifugation of the harvest (i.e. clarification) to a level of 20% or less. Use of a signal sequence comprising "MGRISSGG" (SEQ ID NO: 1) can result in a low % solids remaining after centrifugation of the harvest. For example, the % solids remaining after centrifugation of the harvest may be ≤20%, ≤15%, ≤10%, ≤9%, ≤8%, ≤7%, ≤6%, or ≤5%.

Cell culture as used in the present disclosure is given its broadest meaning, namely the bulk growth of cells in a growth medium. "Fermenting" and "culturing" as used herein means bulk growing the cells in a growth medium. The terms "fermenting" and "culturing" are used interchangeably herein. The exponential phase is a period characterized by cell doubling, therefore bulk growth. The number of new cells appearing per unit time increases significantly and is proportional to the existing population. The cells may be in the exponential growth phase pre-induction. For example, basal expression of the recombinant protein is during the pre-induction exponential phase.

The stationary phase is where the growth rate and death rate of the cells is equal, leading to a linear growth phase. For example, expression of the recombinant protein is induced during the stationary phase. Cells often reach the stationary phase due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of

an inhibitory product such as an organic acid. To maintain a constant biomass in the stationary phase and therefore bulk growth, the cells continue to grow.

Biomass can be estimated by OD^{600}nm. A “high cell density” biomass may be at least OD^{600}nm of 30. For example, a high cell density may refer to an OD^{600}nm of at least 30, at least 40, at least 50, or at least 60. Alternatively, biomass can be measured by dry cell weight. A “high cell density” biomass may be a dry cell weight of at least $10gL^{-1}$. For example, a high cell density may refer to a dry cell weight of at least $10gL^{-1}$, at least $15gL^{-1}$, at least $20gL^{-1}$, or at least $25gL^{-1}$. Alternatively, biomass can be measured by wet cell weight. A “high cell density” biomass may be a wet cell weight of at least $50gL^{-1}$. For example, a high cell density may refer to a wet cell weight of at least $50gL^{-1}$, at least $70gL^{-1}$, at least $85gL^{-1}$, or at least $100gL^{-1}$.

The terms extracellular medium, supernatant, extracellular environment, extracellular space, culture medium, and fermentation medium, are all used herein to describe the external environment of the cells during cell culture, at the point of induction, and at the point of harvest.

The term “induction” or “inducing expression' as used herein, refers to the point at which induction of expression of the recombinant protein is initiated. For example, recombinant protein expression maybe induced by the addition of an inducer, or a change in temperature where induction is temperature dependent. The term “post-induction” is used herein to describe the elapsed time following the point at which induction is initiated.

The term “harvest” is used herein to mean the end of fermentation. Harvest may be at any time point during fermentation that is considered sufficient to end the fermentation process and recover the recombinant protein being expressed. For example, protein recovery begins at the point of harvest. The time of harvest may depend on the optimum concentration of recombinant protein in the supernatant. The recombinant protein may be recovered and purified directly from the extracellular medium of the cell culture at harvest. Alternatively, the recombinant protein may be recovered and purified from the periplasm of the host cell.

"About" as used herein when referring to a measurable value such as an amount, a molecular weight, a temporal duration, and the like, is meant to encompass variations of ±1%, ±0.75%, ±0.5%, ±0.25%, ±0.2%, and ±0.1% from the specified value, as such variations are appropriate to perform the methods described.

Recombinant protein

The recombinant protein may comprise an antigen binding protein, for example a monoclonal antibody, an antibody fragment, or a domain antibody (dAb).

The recombinant protein may comprise a viral protein, a bacterial toxin, a bacterial toxoid, or a cancer antigen.

As used herein a "recombinant protein" refers to any protein and/or polypeptide that can be administered to a mammal to elicit a biological or medical response of a tissue, system, animal or human. The recombinant protein may elicit more than one biological or medical response. Furthermore, the term "therapeutically effective amount" means any amount which, as compared to a corresponding subject who has not received such amount, results in, but is not limited to, healing, prevention, or amelioration of a disease, disorder, or side effect, or a decrease in the rate of advancement of a disease or disorder. The term also includes within its scope amounts effective to enhance normal physiological function as well as amounts effective to cause a physiological function in a patient which enhances or aids in the therapeutic effect of a second pharmaceutical agent.

The term "antigen binding protein" as used herein refers to antibodies, antibody fragments and other protein constructs, such as domains, which are capable of binding to an antigen.

The term "antibody" is used herein in the broadest sense to refer to molecules with an immunoglobulin-like domain. As used herein, "immunoglobulin-like domain" refers to a family of polypeptides which retain the immunoglobulin fold characteristic of antibody molecules, which contain two b-sheets and, usually, a conserved disulphide bond. This family includes monoclonal (for example IgG, IgM, IgA, IgD or IgE), recombinant, polyclonal, chimeric, humanised, bispecific and heteroconjugate antibodies; a single variable domain, a domain antibody, antigen binding fragments, immunologically effective fragments, Fab, F(ab')2, Fv, disulphide linked Fv, single chain Fv, diabodies, TANDABS™, etc.

The phrase "single variable domain" refers to an antigen binding protein variable domain (for example, VH, VHH, VL) that specifically binds an antigen or epitope independently of a different variable region or domain. A "domain antibody" or "dAb" may be considered the same as a "single variable domain".

As used herein "domain" refers to a folded protein structure which retains its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain. By single antibody variable domain or immunoglobulin single variable

domain is meant a folded polypeptide domain comprising sequences characteristic of an antibody variable domain. It therefore includes complete antibody variable domains and modified variable domains, for example in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least in part the binding activity and specificity of the full-length domain.

A domain antibody can be present in a format (e.g., homo- or hetero-multimer) with other variable regions or variable domains where the other regions or domains are not required for antigen binding by the single immunoglobulin variable domain (i.e., where the immunoglobulin single variable domain binds antigen independently of the additional variable domains).

The domain antibody (dAb^{TM}) may be a human antibody variable domain. The dAb^{TM} may be of human origin. In other words, the dAb^{TM} may be based on a human Ig framework sequence.

As used herein, the term “antigen binding site” refers to a site on an antigen binding protein which is capable of specifically binding to an antigen, this may be a single domain, or it may be paired VH/VL domains as can be found on a standard antibody. Single-chain Fv (ScFv) domains can also provide antigen-binding sites.

The antigen binding protein may comprise additional antigen binding sites for different antigens. For example, the antigen binding protein may have specificity for more than one antigen, for example two antigens, or for three antigens, or for four antigens.

The antigen binding protein may consist of, or consist essentially of, an Fc region of an antibody, or a part thereof, linked at each end, directly or indirectly (for example, via a linker sequence) to a binding domain. Such an antigen binding protein may comprise two binding domains separated by an Fc region, or part thereof. By separated is meant that the binding domains are not directly linked to one another, and may be located at opposite ends (C and N terminus) of an Fc region, or any other scaffold region.

The antigen binding protein may comprise two scaffold regions each bound to two binding domains, for example at the N and C termini of each scaffold region, either directly or indirectly via a linker. Each binding domain may bind to a different antigen.

The antigen binding protein may take the protein scaffold format of a mAbdAb. "mAbdAb" and “dAbmAb" are used interchangeably, and are intended to have the same meaning as used herein. Such antigen-binding proteins comprise a protein scaffold, for

example an Ig scaffold such as IgG, for example a monoclonal antibody, which is linked to a further binding domain, for example a domain antibody. A mAbdAb has at least two antigen binding sites, at least one of which is from a domain antibody, and at least one is from a paired VH/VL domain.

Domain antibodies can exist and bind to target in monomeric or multimeric (e.g. dimeric) forms, and can be used in combination with other molecules for formatting and targeting approaches. For example, an antigen-binding protein having multiple domains can be made in which one of the domains binds to serum proteins such as albumin. Domain antibodies that bind serum albumin (AlbudAbs) are known and can provide the domain fusion partner an extended serum half-life in its own right.

dAbs may also be conjugated to other molecules, for instance in the form of a dAb-conjugate or a dAb-fusion with other molecules e.g. a drug, another protein, an antibody molecule or an antibody fragment. For example a dAb™ can be present as a formatted dAb™, e.g. the dAb™ can be present as a dAb-Fc fusion or conjugate. Alternatively, the formatted dAb™ can be present as a mAbdAb. The dAb™ may be present as a fusion or conjugate with half life extending proteins or polypeptides, for example, a further dAb™ which binds to serum albumin (AlbudAb), or to a half life extending chemical moiety such as polyethyleneglygol (PEG). The dAb™ may be present as a fusion or conjugate with further therapeutic or active molecules.

As used herein, "drug" refers to any compound (for example, a small organic molecule, a nucleic acid, a polypeptide) that can be administered to an individual to produce a beneficial therapeutic or diagnostic effect through binding to and/or altering the function of a biological target molecule in the individual. The target molecule can be an endogenous target molecule encoded by the individual's genome (e.g., an enzyme, receptor, growth factor, cytokine encoded by the individual's genome) or an exogenous target molecule encoded by the genome of a pathogen. The drug may be a dAb™ or mAb.

A "dAb conjugate" refers to a composition comprising a dAb™ to which a drug is chemically conjugated by means of a covalent or noncovalent linkage. Preferably, the dAb™ and the drug are covalently bonded. Such covalent linkage could be through a peptide bond or other means such as via a modified side chain. The noncovalent bonding may be direct (e.g., electrostatic interaction, hydrophobic interaction) or indirect (e.g., through noncovalent binding of complementary binding partners (e.g., biotin and avidin), wherein one partner is covalently bonded to drug and the complementary binding partner is covalently bonded to the dAb™). When complementary binding partners are employed, one of the binding partners can be covalently bonded to the drug directly or through a

suitable linker moiety, and the complementary binding partner can be covalently bonded to the dAb™ directly or through a suitable linker moiety.

As used herein, "dAb fusion" refers to a fusion protein that comprises a dAb™ and a polypeptide drug (which could be a polypeptide, a dAb™ or a mAb). The dAb™ and the polypeptide drug are present as discrete parts (moieties) of a single continuous polypeptide chain.

Thus the methods of the disclosure may be applied to one or more of: a therapeutic protein, a monoclonal antibody (mAb), a domain antibody (dAb™), a dAb™ conjugate, a dAb™ fusion, a mAbdAb, or any other antigen binding protein described herein.

In an embodiment, the antigen binding protein is a dAb™ which interferes with TNFα signalling. In an embodiment, the dAb™ neutralises TNFα. In an embodiment, the dAb™ specifically binds to TNFα or a TNFα receptor. In an embodiment, the dAb™ specifically binds TNFR1. In an embodiment, the antigen binding protein is VH dAb™ (anti-TNFR1) / DOM0101) of SEQ ID NO: 53 (DOM0101).

Expression of Protein: Host cell

Suitable host cells include microbial cells such as Gram-negative bacteria for example *Escherichia coli* (e.g. W3110, and BL21), *Pseudomonas*, and *Salmonella*. In a specific embodiment the host cell is *Escherichia coli*. In an embodiment the *E. coli* strain is W3110.

A vector comprising a recombinant nucleic acid molecule encoding the signal sequence and the recombinant protein is also described herein. Such vectors are employed to genetically engineer cells to express the desired protein product. The vector may be an expression vector comprising one or more expression control elements or sequences that are operably linked to the signal sequence and the recombinant nucleic acid. Examples of vectors include plasmids and phagemids.

Suitable expression vectors can contain a number of components, for example, an origin of replication, a selectable marker gene, one or more expression control elements, such as a transcription control element (e.g. promoter, enhancer, terminator) and/or one or more translation signals, a signal sequence or leader sequence. Expression control elements and a signal sequence, if present, can be provided by the vector or other source. For example, the transcriptional and/or translational control sequences of a cloned nucleic acid encoding an antibody chain can be used to direct expression.

A promoter can be provided for expression in a desired cell. Promoters can be constitutive or inducible. For example, a promoter can be operably linked to a nucleic acid

encoding an antibody, antibody chain or portion thereof, such that it directs transcription of the nucleic acid. A variety of suitable promoters for Gram negative bacteria (*e.g.*, lac, tac, trp, phoA, lambdapL, T3, T7 (T7A1, T7A2, T7A3) promoters for *E. coli*) may be used. Operator sequences which may be employed include lac, gal, deo and gin. One or more perfect palindrome operator sequences may be employed. In an embodiment expression of the recombinant protein of the disclosure is under the control of an inducible promoter. For example, *E.coli* lac, tac, and trc promoters are inducible with lactose or the non-hydrolyzable lactose analogue, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the phoA, trp and araBAD promoters are inducible by phosphate, tryptophan and L-arabinose respectively.

In addition, expression vectors typically comprise a selectable marker for selection of cells carrying the vector, and, in the case of a replicable expression vector, an origin of replication. Genes encoding products which confer antibiotic or drug resistance are common selectable markers and may be used (e.g. lactamase gene (ampicillin resistance), *Tet* gene for tetracycline resistance). Dihydrofolate reductase marker genes permit selection with methotrexate in a variety of cells.

An expression vector as described in WO2007/088371 (for example pAVE037, pAVE007, or pAVE011) may be used to express the protein. In an embodiment, the vector is pAVE011. Alternatively, a vector such as pJExpress401 may be used to express the protein.

Example alternative expression vectors and methodologies (e.g. for use with CHO, PerC6 etc) are also known.

The host cell comprises the recombinant nucleic acid molecule or vector described above.

Host cell cultures of the present disclosure may be cultured in any medium that supports the host cell growth and expression of the recombinant protein. Such media are well known to those skilled in the art.

Protein Targeting

Although expression of a recombinant protein takes place in the cytoplasm, the final location of the recombinant protein may be cytoplasmic, periplasmic or extracellular depending on the nature of the recombinant protein, the host cell used and the fermentation conditions used.

The use of the signal sequences in the present invention may be with respect to a recombinant protein that is targeted to the periplasm of the cell (e.g. in Gram negative bacterium).

In Gram-negative bacteria, some secreted proteins are exported across the inner and outer membranes in a single step via the type I, type III, type IV or type VI secretion pathways, whereas other proteins are first exported into the periplasm via the universal Sec or Tat pathways and then translocated across the outer membrane mainly via the type II or type V machinery. The type II system involves a two-step process in which a premature protein containing a Sec leader sequence is exported to the periplasm using the Sec pathway. The signal sequence is removed by proteolysis resulting in a mature, processed protein being present in the periplasm and whether or not the protein is secreted to the culture medium highly depends on the characteristics of leader sequence, protein, cell and culture conditions. Also in the case of cell lysis (autolysis) it can be assumed that the majority of the protein in the culture medium originates from the periplasm and therefore is processed. The recombinant protein may be actively secreted into the culture medium via the signal sequence; or passively from the periplasm to the culture medium via other cellular pathways known in the art.

Processing of the signal sequence includes cleavage and removal of the signal sequence from the protein. However, some amino acids of the signal may remain at the N-terminus of the protein, such that the signal sequence is not properly processed. The signal sequence may be 90% or more processed, such that 10% or less of the sequence remains at the N-terminus of the protein. The signal sequence may be at least 91, 92, 93, 94, 95, 96, 97, 98, or 99% processed. The signal sequence may about 100% processed, such that none remains at the N-terminus of the protein following passage through the secretory pathway of the cell.

The signal sequence may be a periplasmic targeting signal sequence, e.g. an N-terminal periplasmic targeting sequence. Signal sequences to direct proteins to the periplasm or extracellular environment are known in the art. The signal sequence may comprise a heterologous signal sequence.

Harvest

Harvest is the end of fermentation. Harvest may be at any time point during fermentation that is considered sufficient to end the fermentation process and recover the recombinant protein being expressed. Harvest may occur between 10 and 160 hours post induction. For example, harvest may occur between 15 and 60 hours post induction.

Harvest may occur 24, 48, or 55 hours post induction. At harvest, the solid content of the microbial cell population may be between 5-30% Wet Cell Weight (WCW).

The fermentor volume may be:

(i) about 10,000 litres; about 5,000 litres; about 2,000 litres; about 1,000 litres; about 500 litres; about 125 litres; about 50 litres; about 20 litres; about 10 litres; about 5 litres; or

(ii) between 5 and 10,000 litres; between 10 and 5,000 litres; between 20 and 2,000 litres; between 50 and 1,000 litres.

The harvest may comprise cells that have naturally lysed, also known as auto-lysis. For example, 1-50% of the cells in the harvest may have undergone autolysis. Alternatively, 20-50%; or 30-50%; or 40-50% of the cells in the harvest have autolysed. Alternatively, 10% or more; 20% or more; 30% or more; 40% or more; or 50% or more of the cells in the harvest have autolysed. Autolysis may be indirectly determined by DNA concentration in a clarified harvest, or by capacitance.

Harvest may include the optional step of emptying the fermentor of the cells and extracellular media (i.e. the cell culture or broth).

Optional pre-treatment of harvest

Dependent on host cell and recombinant protein, the pre-treatment of the harvest is a method of conditioning the harvest. This step may be carried out in the fermentor, or after the harvest has been removed from the fermentor. Pre-treatment includes: thermally, mechanically or chemically lysing the harvest (for example by homogenisation, freeze-thaw, lysis); and periplasmic extraction. At least one periplasmic extract may be extracted using methods known in the art. Alternatively if sufficient product is already present in the extracellular environment then such pre-treatment may not be required.

Clarification

Clarification is the process to remove solid particulates. Clarification can lower the burden on subsequent chromatographic steps during purification. Typical clarification steps comprise a settling step – also known as sedimentation (e.g. by gravity), and/or a centrifugation step, and/or a filtration step.

The centrifugation step may be continuous centrifugation (e.g. with a continuous feed zone). The centrifuge may in itself be operating “batch” or “intermittently” or “continuously” with respect to discharging the solids. For example, a tubular bowl centrifuge may be used as the continuous centrifugation step.

Purification of the recombinant protein

The recombinant protein may be recovered directly from the culture medium. Recovery of the recombinant protein is followed by purification to ensure adequate purity of the recombinant protein. One or more chromatography steps may be used in purification, for example one or more chromatography resins; and/or one or more filtration steps. For example affinity chromatography using resins such as protein A or L may be used to purify the recombinant protein. Alternatively, or in addition to, an ion-exchange resin such as a cation-exchange may be used to purify the recombinant protein.

Examples

A range of native, endogenous *E. coli* N-terminal signal sequences (*SS*) were studied for domain antibody (dAb) expression from an *E. coli* W3110 host strain. Two sequences with promising attributes, *YceISS* and *IvySS* were taken forward for bioreactor studies with comparison against a currently used signal sequence, *OmpASS.* For the bioreactor conditions studied, the use of *OmpASS* yielded a high product titre in a short fermentation time but with a highly viscous broth, which was predicted by ultra scale-down studies to be challenging to clarify by full-scale centrifugation. The use of *YceISS* led to performance comparable to that for *OmpASS* in terms of titre and viscosity and difficulty of centrifugation, while the use of *IvySS* led to a low viscosity broth which was relatively easy to centrifuge, and a high product titre but achieved over a longer fermentation time. Codon optimisation of the *IvySS* led to similarly low viscosity broth suitable for centrifugation and a high titre but now over shorter fermentation times comparable to that for the control using *OmpASS*. The improvements achieved using the *IvySS* or its optimised versions appear to be related to the reduced basal expression of dAb i.e. before induction. This reduced expression is believed to be due to the presence of an eight codon cassette from the N-terminus of the signal sequence, which if removed resulted in high viscosity broths similar to that obtained using the *OmpASS*. The relationship between basal expression and broth viscosity is investigated.

Materials and Methods

Molecular cloning of secretion cassettes. *E. coli* strains One Shot™ TOP10 (Life Technologies, California, USA) and W3110 (ATCC 27325) were used as a host strains for cloning and recombinant protein expression respectively. Oligonucleotides (Table 2) designed for generation of dAb secretion cassettes with various upstream signal sequences were constructed to specification (Invitrogen, Paisley, UK). Reagents used were restriction enzymes (*Nde*I and *Xho*I), T4 DNA Ligase (New England Biolabs UK Ltd., Hertfordshire, UK) and DNA polymerase (Phusion™ flash high-fidelity PCR master mix, Finnzymes Oy, Espoo, Finland). The pAVEway™ expression vector, pAVE011 (here on

denoted as p11), containing the tetracycline resistance gene, *tetA*, and an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7A3 promoter (WO2007088371; Fujifilm Diosynth Biotechnologies UK Ltd., Billingham, UK) was used as a backbone for dAb secretion cassettes.

Table 2. Oligonucleotides used for construction of *dAb* gene constructs with different signal sequences. Restriction sites *Nde*I (CATATG) and *Xho*I (CTCGAG) are underlined.

SEQ ID NO:	Oligo Name	Oligonucleotide Sequence (5' to 3')
2	IvySS fwd1	ctctggtcatcgctaccagtgcaatggcggaagtacaactgctggagag
3	IvySS fwd2	tgatgtttaaggcaataacgacagtcgccgctctggtcatcgctaccagtg
4	IvySS fwd3	gaccatatgggcaggataagctcgggaggaatgatgtttaaggcaataacg
5	YceISS fwd1	ttctctgccggttcagcggttgccgaagtacaactgctggagag
6	YceISS fwd2	ggtttaaccttcgcgtccctgatgttctctgccggttcagc
7	YceISS fwd2	gaccatatgaaaaaaagcctgcttggtttaaccttcgcgtccctg
8	YgiWSS fwd1	gtgcagcgcaccggtgatggcagaagtacaactgctggagag
9	YgiWSS fwd2	cagtaatcgcagtaatggccctgtgcagcgcaccggtgatgg
10	YgiWSS fwd3	gaccatatgaaaaaattcgcagcagtaatcgcagtaatggccctgtg
11	YncESS fwd1	gtttcatcattcagtacgcaggccgaagtacaactgctggagag
12	YncESS fwd2	ttactgttaggttcattgcttgttgtttcatcattcagtacgcaggc
13	YncESS fwd3	ttttcatcgcgcctgcgtggttcattactgttaggttcattgcttgttg
14	YncESS fwd4	gaccatatgcatttacgtcatctgttttcatcgcgcctgcgtgg
15	FimASS fwd1	cctcagttctacagcggctctggccgaagtacaactgctggagag
16	FimASS fwd2	caatcgttgttctgtcggctctgtccctcagttctacagcggctctgg
17	FimASS fwd3	gaccatatgaaaattaaaactctggcaatcgttgttctgtcggctctg
18	YehZSS fwd1	ggcagccgtgagcctgccgctacaagcagaagtacaactgctggagag
19	YehZSS fwd2	ctgggcaggttcactggttatgttggcagccgtgagcctgccgctacaag
20	YehZSS fwd3	gaccatatgccactcttaaagctctgggcaggttcactggttatg
21	YcdOSS fwd1	gttttcttctgcttttatggctaacgccgaagtacaactgctggagag
22	YcdOSS fwd2	acgcattgcagttgagcgtggctgcgctgttttcttctgcttttatggctaac
23	YcdOSS fwd3	gaccatatgaccattaacttccgccgtaacgcattgcagttgagcgtg
24	IvyOpt1SS fwd1	gctctggttattgctactagcgctatggctgaagtacaactgctggagagc

25	IvyOpt1SS fwd2	atgatgttcaaagcaatcaccacagtggcagctctggttattgctactagcg
26	IvyOpt1SS fwd3	gaccatatgggacgtatttcttcgggcggaatgatgttcaaagcaatcaccacag
27	IvyOpt2SS fwd1	gctctggttattgcgacctctgctatggcagaagtacaactgctggagagcg
28	IvyOpt2SS fwd2	atgatgttcaaagcaatcactaccgttgcagctctggttattgcgacctc
29	IvyOpt2SS fwd3	gaccatatgggtcgtatctcttccggcggtatgatgttcaaagcaatcactac
30	IvyTruncSS fwd	gaccatatgatgtttaaggcaataacgacagtcgccgctctg
31	Ivy'OmpASS fwd	gaccatatgggcaggataagctcgggaggaatgaagaaaactgctatcgc
32	dAb reverse	cttactcgagtcattagctgcttacggtgaccagag

A selection of seven endogenous *E. coli* signal sequences (Table 3) were genetically fused upstream of a gene encoding a V_H domain antibody against TNFR1 with a molecular weight of ~13.1 kDa (here on denoted as dAb, SEQ ID NO: 53). Up to four sequential PCR reactions were used to extend the upstream end of the *dAb* with the DNA for the desired signal sequence. This required 3 or 4 forward oligos (one for each sequential PCR reaction), reverse oligo SEQ ID NO: 32 (see Table 2) and the p11 expression plasmid as an initial template (pDAB01, see Table 3). PCR reactions contained the appropriate forward (fwd) oligo and reverse (rev) oligo SEQ ID NO: 32 (Table 2), DNA template and Phusion™ flash high-fidelity PCR master mix. Thermal cycling was performed with the following conditions: 98°C for 10 s followed by 30 cycles of 98°C for 1 s, 55°C for 5s and 72°C for 15s, and a final incubation at 72°C for 1 min. After each round of PCR, the product was purified using column chromatography (QIAquick™ PCR Purification Kit, QIAGEN Ltd., Manchester, UK) and used as a DNA template for the next sequential PCR reaction until the signal sequence-*dAb* fusion cassette flanked by *Nde*I and *Xho*I restriction sites was generated. Secretion cassettes were digested with *Nde*I and *Xho*I and ligated into an *NdeI-XhoI* digested p11 expression vector using T4 DNA Ligase, then transformed by heat shock into *E. coli* One Shot™ TOP10 cells and selected on vLB agar tetracycline plates. Plasmids pDAB02 to pDAB08 (Table 3) were confirmed by DNA sequencing, then transformed into *E. coli* W3110 cells for protein expression studies. Cells banks (glycerol stocks, 20% v/v) of each prepared construct were stored at -80°C.

Table 3. Expression plasmids and *E. coli* signal sequenced used for dAb expression studies. [a] Signal sequence name and [no. of codons]; [b] Signal sequences amino acid sequence with the C-terminal cleavage site at the end of the sequence. [c] Codon adaptation index (CAI) for each signal sequence was analysed using EMBOSS suite referenced against the codon usage for *E. coli* class II (high expressing) genes (Eecoli_high.cut).

Plasmid Name	[a] Signal Sequence	[b] Amino Acid Sequence	Nucleotide Sequence (SEQ ID NO)	[c] CAI
pDAB01	*OmpASS* [21]	MKKTAIAIAVALAGFATVAQA (SEQ ID NO: 33)	atgaagaaaactgctatcgctattgcggttgctctggcaggttttgccacggttgcgcaggcc (SEQ ID NO: 41)	0.701
pDAB02	*IvySS* [28]*	MGRISSGGMMFKAITTVAALVIATSAMA (SEQ ID NO: 34)	atgggcaggataagctcgggaggaatgatgtttaaggcaataacgacagtcgccgctctggtcatcgctaccagtgcaatggcg (SEQ ID NO: 42)	0.267
pDAB03	*YceISS* [22]	MKKSLLGLTFASLMFSAGSAVA (SEQ ID NO: 35)	atgaaaaaaagcctgcttggtttaaccttcgcgtccctgatgttctctgccggttcagcggttgcc (SEQ ID NO: 43)	0.639
pDAB04	*YgiWSS* [20]	MKKFAAVIAVMALCSAPVMA (SEQ ID NO: 36)	atgaaaaaattcgcagcagtaatcgcagtaatggccctgtgcagcgcaccggtgatggca (SEQ ID NO: 44)	0.808
pDAB05	*YncESS* [30]	MHLRHLFSSRLRGSLLLGSLLVVSSFSTQA (SEQ ID NO: 37)	atgcatttacgtcatctgttttcatcgcgcctgcgtggttcattactgttaggttcattgcttgttgtttcatcattcagtacgcaggcc (SEQ ID NO: 45)	0.316
pDAB06	*FimASS* [23]	MKIKTLAIVVLSALSLSSTAALA (SEQ ID NO: 38)	atgaaaattaaaactctggcaatcgttgttctgtcggctctgtccctcagttctacagcggctctggcc (SEQ ID NO: 46)	0.606
pDAB07	*YehZSS* [23]	MPLLKLWAGSLVMLAAVSLPLQA (SEQ ID NO: 39)	atgccactcttaaagctctgggcaggttcactggttatgttggcagccgtgagcctgccgctacaggcg (SEQ ID NO: 47)	0.377
pDAB08	*YcdOSS* [26]	MTINFRRNALQLSVAALFSSAFMANA (SEQ ID NO: 40)	atgaccattaacttccgccgtaacgcattgcagttgagcgtggctgcgctgtttcttctgcttttatggctaacgcc (SEQ ID NO: 48)	0.652

*The amino acid sequence is as for Ivyss. The codon sequence at position 23 has been altered from gcc to gct.

Table 4. Expression plasmids containing modified *E. coli* Ivy signal sequences to investigate the effect of codon adaptation index (CAI) on the expression rate of a model dAb and subsequent effects on broth viscosity during high cell density fed-batch fermentation. [a] Signal sequence name and [no. of codons]. [b] Codon usage index for each signal sequences was analysed using EMBOSS suite referenced against the codon usage for *E. coli* class II (high expressing) genes (Eecoli_high.cut).

Plasmid Name	[a] Signal Sequence	Description	Nucleotide Sequence	[b] CAI
pDAB021	*IvyOpt1SS* [28]	Full codon optimisation with reference to *E. coli* genes	Atgggacgtatttcttcgggcggaatgatgttcaaagcaatcacc acagtggcagctctggttattgctactagcgctatggct (SEQ ID NO: 49)	0.580
pDAB022	*IvyOpt2SS* [28]	Full codon optimisation with reference to class II genes only	atgggtcgtatctcttccggcggtatgatgttcaaagcaatcacta ccgttgcagctctggttattgcgacctctgctatggca (SEQ ID NO: 50)	0.899
pDAB023	*IvyTruncSS* [20]	First 8 *IvySS* codons removed	atgatgtttaaggcaataacgacagtcgccgctctggtcatcgct accagtgcaatggcg (SEQ ID NO: 51)	0.403
pDAB011	*Ivy'OmpASS* [29]	First 8 *IvySS* codons + *OmpASS* MGRISSGGMKKTAIAIAVALAGFA TVAQA (SEQ ID NO: 55)	atgggcaggataagctcgggaggaatgaagaaaactgctatc gctattgcggttgctctggcaggttttgccacggttgcgcaggcc (SEQ ID NO: 52)	0.396

Table 5. Effect of N-terminal signal peptide sequence on the level of basal dAb expression, broth viscosity and initial productivity during high cell density *E. coli* fermentation. [a] Initial productivity is the total dAb expressed in the first 24 h post-induction (Figs 2E & 4E). [b] The N-terminal cassette comprises the codons for the first eight amino acids from the IvySS.

Output	Signal sequences [CAI, No. of codons, CAI/No.]		
	No Cassette	[b] Cassette	
	OmpASS [0.702, 21, 0.033] *YcelSS* [0.639, 21, 0.029] *IvyTruncSS* [0.403, 20, 0.020]	*IvySS* [0.267, 28, 0.009] *Ivy'OmpASS* [0.396, 29, 0.013]	*IvyOpt1SS* [0.580, 28, 0.021] *IvyOpt2SS* [0.899, 28, 0.032]
Basal Expression	High	Low	Low
Viscosity (Pre-Induction)	High	Low	Low
[a] Initial Productivity	High	Low	High

Secretion cassettes for dAb expression plasmids pDAB021 and pDAB022 (Table 4) containing codon optimised Ivy signal sequences, *IvyOpt1SS* and *IvyOpt2SS*, were generated by PCR amplification of the gene coding for dAb from pDAB01 (as described above) using fwd oligos 23-25 and 26-28 respectively and reverse oligo SEQ ID NO: 32 (Table 2). The secretion cassette for pDAB023 (Table 4) containing a truncated Ivy signal sequence (*IvyTruncSS*) upstream of *dAb* was generated by PCR amplification using pDAB02 as a template (Table 3) and fwd oligo 29 and rev oligo 31. The secretion cassette for pDAB011 encoded for the dAb with the upstream signal sequence *Ivy'OmpASS* composed of the first 8 codons of the *IvySS* fused to *OmpASS*. This was generated by amplifying the gene encoding the *OmpASS*-dAb from pDAB01 (Table 3) using fwd oligo 30 and rev oligo SEQ ID NO: 32. All secretion cassettes were subsequently ligated into the p11 vector at *Nde*I/*Xho*I and transformed into the *E. coli* W3110 strain.

The codon adaptation index (CAI) (Sharp and Li 1987) for each signal sequence was determined using EMBOSS suite (Rice et al. 2000) referenced against the codon usage for class II (high expressing) genes only (Eecoli_high.cut). *IvySS* was codon optimised for expression in *E. coli* using a codon adaptation algorithm (Leto Software, version 1.0.26, Entelechon GmbH, Regensburg, Germany). The codon (Leto) threshold was set at 70% with reference to all *E. coli* K12 genes and also for *E. coli* K12 class II (high expressing) genes only to generate *IvyOpt1SS* and *IvyOpt2SS* respectively (Table 4).

Fermentation. All media and reagents were purchased from Sigma-Aldrich (Dorset, UK) unless specified. Inoculum and 24-deep well plate (24 dwp) cultivation was performed in Luria Bertani (vLB) media (pH 7.0): 10 $g.L^{-1}$ Difco™ Select Soytone (Becton Dickinson and Co. (BD), New Jersey, USA), 5 $g.L^{-1}$ Bacto™ Yeast Extract (BD) and 5 $g.L^{-1}$ NaCl. 10x concentrated glycerol boost (pH 7.0) for 24 dwp cultures contained: 70 $g.L^{-1}$ glycerol, 50 $g.L^{-1}$ Yeast Extract (BD), 25 $g.L^{-1}$ $(NH_4)_2SO_4$ and 1.0 $\mu g.mL^{-1}$ thiamine. High cell density fermentation was performed in a complex medium as described elsewhere (Voulgaris et al. 2015). All fermentation media (excluding TB/SB feed) was supplemented with 15 $\mu g.mL^{-1}$ tetracycline (Tet) for selectivity of *E. coli* clone containing the p11 expression plasmid.

For deep well plate expression inoculum cultures in vLB media (20 mL) were prepared in 125 mL Erlenmeyer, baffled, vent cap shake flasks (Corning Life Sciences, Amsterdam, Netherlands) with 0.5% v/v *E. coli* W3110 transformed with a pDAB expression plasmid. Cultures were incubated overnight at 23°C, 230 rpm in an orbital shaker with a 25 mm offset (Climo-shaker ISF1-X, Kühner Shaker Ltd., Derbyshire, UK) to an OD_{600nm} of 1 - 2. This was used to inoculate fresh vLB media to an OD_{600nm} of 0.05 and was dispensed into pyramid bottom square shaped wells (2 mL working volume) of 24-deep well microtitre

plates (24 dwp; Porvair Sciences Ltd., Wrexham, UK) and covered with a high cell density gas-permeable lid system (Applikon Biotechnology, Tewkesbury, UK). Plates were incubated at 37 °C, 230 rpm to OD_{600nm} 0.7 - 1.0 and then dAb formation was induced with 0.1 mM IPTG with simultaneous addition of 10x glycerol boost (see above for recipe). Cultures were incubated for a further 24 h at 23 °C, 230 rpm. Harvest cultures (2 mL) were pelleted (16100 x g for 10 min) and the broth supernatant extracted. Pellets were resuspended in 1 mL 0.1 M PBS and centrifuged again as before. Broth supernatant and pellets were stored at -20°C.

For Bioreactor expression inoculum cultures in vLB media (200 mL) were prepared with 0.5% v/v glycerol stocks in 500 mL Erlenmeyer, baffled, vent cap shake flasks (Corning Life Sciences). Cultures were incubated at 37 ^{0}C, 230 rpm in an orbital shaker for 4 - 5 h until an OD_{600nm} of 1 - 2 and used at 0.05% v/v to inoculate a 1 L working volume reactor (SR1000DLL bioreactor, vessel dia 100 mm, aspect ratio 2.4:1, overhead driven triple Rushton 6-blade, 46 mm dia, impellers, DASGIP AG, Jülich, Germany) containing the complex medium mentioned above. The reactor was maintained at: 30°C, pH 7.0 ±0.05 (using 25% v/v H_3PO_4 and 30% v/v NH_4OH); Dissolved Oxygen (DO) 30±5% (by cascade control using: (1) impeller speed (400 - 1200 rpm); (2) gas flow rate (1 - 2 vvm) and (3) oxygen content (21 - 100%)). Feed medium was automatically added at 6.0 $mL.L^{-1}.h^{-1}$ starting 10 min after the DO spike indicating complete glycerol consumption. Production of dAb was induced with the addition of 0.2 mM IPTG once the culture had reached an OD_{600nm} of 75±3 (corresponding to a biomass of approximately 25 dcw $g.L^{-1}$) and the feed rate was reduced to 3.6 $mL.L^{-1}.h^{-1}$ for the rest of the fermentation. Real-time values of pH, dissolved oxygen, agitation speed, temperature, air-flow rate, oxygen percentage, oxygen uptake rate, carbon dioxide evolution rate were recorded (DASGIP Control, Jülich, Germany). The rheological characteristic of the fresh cell broth was measured (DV-II+ Programmable Viscometer fitted with either ULA, 1.233N or SSA, 1.32N spindle, Brookfield Engineering Laboratories Inc., Massachusetts, USA) over increasing and decreasing shear sweeps (1.3 to 110 s^{-1} in five increments with 30s hold at each increment) at 25°C. Rheology is used as an indication of broth processability e.g. by centrifugation.

Cell broth processing. An ultra scale-down shear device (20 mL stainless steel chamber, 50 mm dia x 10 mm height, fitted with a stainless steel rotating disc, 40 mm dia x 0.1 mm thick, 12000 rpm, constructed in the UCL mechanical workshop) was used to expose cell broth samples to a maximum energy dissipation rate (ε) of 0.53 $x10^6$ $W.kg^{-1}$ for 20 s (Chatel et al. 2014a). Such an ε value typically mimics that which occurs in the feed zone of a high shear continues-flow industrial scale centrifuge. Samples (2 mL of sheared or non-sheared broth) were then centrifuged (3400 x g for 7 min at 21°C, Centrifuge 5415R,

Eppendorf, Cambridge, UK) and the supernatants (0.8 mL) were recovered. These were characterized in terms of the solids remaining by measurement at OD_{600nm} corrected against a baseline OD_{600nm} value for a well-clarified sample (16100 x g for 30 min). The centrifugation conditions were characterized in terms of the group V/tΣ, where V is volume centrifuged (2.0 mL), t is time of centrifugation (420 s) and Σ is equivalent settling area of the centrifuge tube for the particular rotational speed and geometry of centrifuge head (= 0.164 m^2 – see Chatel et al. 2014a for details of calculation).

Analytical methods. Cell pellets were thawed and resuspended in 1 mL (24 dwp samples) or 4 mL (1 L bioreactor samples) of 50 mM Tris-HCl pH 7.5. 1 mL of each resuspended pellet was lysed (Soniprep 150 Plus Ultrasonic disintegrator fitted with an exponential microprobe, part no. 38121-114A, MSE (UK) Ltd., London, UK) using two pulses of 30 s on and 10 s off at amplitude 8.0. The lysed whole cell sample was then centrifuged at 16100 x g for 20 min and the supernatant recovered. Fermentation broth samples and whole cell lysates were then filtered using a 0.22 µm filter plate centrifuged at 3000 x g for 30 min (GHP membrane, AcroPrep™ 96 Filter Plate, Pall Life Sciences, Portsmouth, UK). The expression of dAb from *E. coli* 24 dwp cultures was analysed by SDS-PAGE. Samples were reduced, denatured and loaded on a SDS-PAGE gel (NuPAGE® 4-12% Bis-Tris, Invitrogen) with gel electrophoresis performed in 1x MES running buffer (Invitrogen) as per the manufacturer's instructions. Protein was detected by Coomassie staining and product was quantified using densitometry software (Image Lab version 3.0, Bio-Rad Laboratories). For high cell density *E. coli* fermentation, dAb product titre in the broth supernatant and whole cell lysate were determined by Protein A HPLC (HP1100 with a chilled autosampler, Agilent Technologies UK Limited, Cheshire, UK) fitted with a 1 mL HiTrap MabSelect Xtra column (GE Healthcare Life Science, Buckinghamshire, UK). The column was equilibrated and washed with 0.1 M PBS pH 7.3 and 20 mM HCl pH 1.8 was used for product elution (protein concentration was monitored at 280 nm). Concentration of dAb was determined using a standard curve. The molecular weight of the purified dAb was determined using liquid chromatography time-of-flight mass spectrometry (GSK in-house method using Agilent HP1100, Agilent Technologies UK Ltd., coupled to a Micromass LCT mass spectrometer, Waters, Massachusetts, USA, equipped with an electrospray ionization (ESI) probe and was controlled using MassLynx, version 4.1, Waters, software). Double-stranded DNA was measured using fluorometry (Qubit™ dsDNA BR Assay kit, Life Technologies) as per the manufacturer's instructions.

Example 1

Figure 1 shows the final biomass levels and SDS-PAGE gels, detailing the expression of dAb in terms of extracellular and intracellular location, of an initial screen of N-terminal signal sequences.

The effect of different N-terminal signal sequences on the expression of dAb from *E. coli* W3110 in 24 dwp fermentations is shown in Figure 1. Key: B- dAb located extracellularly (supernatant); C- dAb located intracellularly (whole cell lysate: periplasmic + cytosolic). B + C - total dAb, B/(B + C) - proportion released. Summation of B and C allows visual estimation of total dAb formed. Samples were taken 24 h post-induction, reduced and bands visualised by Coomassie stained SDS-PAGE. 'Std' denotes dAb standard at 0.5 $mg.mL^{-1}$ concentration.

The use of the *OmpA* signal sequence (*OmpASS*), *IvySS* and *YceISS* results in high dAb expression levels per unit volume of broth (broth = cells + supernatant, i.e. extracellular medium), while the use of *YgiWSS*, *YncESS*, *FimASS*, *YehZSS* and *YcdOSS* signal sequences gives moderate expression levels. *OmpASS*, *IvySS*, *YceISS* and *FimASS* result in high intracellular product levels per unit OD_{600nm}, while *YgiWSS*, *YncESS*, *YehZSS* and *YcdOSS* result in moderate levels. There is some positive correlation between the intracellular product level per unit OD_{600nm} and product release to the supernatant for *OmpASS*, *YceISS*, *YgiWSS*, *FimASS*, and *YehZSS*. The use of *YceISS*, *YncESS* and *YcdOSS* appears to lead to high release levels and for *YceISS*, this plus the high titre achieved, makes it one candidate for further study. *IvySS* exhibits a relatively low release to the supernatant and a high OD_{600nm}, which might signify further potential for dAb formation. This plus the resulting high total product and relatively low proportional release of dAb to broth identifies it as the second candidate for further study. These two strains are carried forward for more detailed study of growth in bioreactors against the use of *OmpASS* as a control.

Example 2

The effect of using *OmpASS*, *YceISS* and *IvySS* on the fed-batch fermentation of *E. coli* W3110 is described in Figure 2. The fermentation was monitored in the post-induction phase via OD_{600nm} as an indication of cell growth, dsDNA release as an indication of lysis, extracellular and intracellular dAb as an indication of productivity and product location, rheology as an indication of broth processability e.g. by centrifugation.

Figure 2 shows post-induction fermentation profiles for high cell density *E. coli* W3110 fed-batch cultures. N-terminal signal sequence and basal (pre-induction) expression level: (Δ), *OmpASS*, 0.30 $g.L^{-1}$, (□) *YceISS*, 0.64 $g.L^{-1}$ or (◊), *IvySS*, 0.061 $g.L^{-1}$. All results are taken post-induction of dAb formation with IPTG. (A) Biomass was

estimated by OD_{600nm}; (B) Extracellular soluble dsDNA concentration; dAb yield was determined (C) intracellularly (whole cell lysate), (D) extracellularly (supernatant), as (E) total dAb formed (intracellular + extracellular) and as (F) percentage of total dAb in the extracellular space. Broth viscosity, μ, is described in terms of a power law relationship, $\mu = K \gamma^{n-1}$ where γ is shear rate, (G) K is the flow consistency index, and (H) n is the flow behaviour index. Results were taken from replicated fermenters (*n* = 2-4) averaged and error bars added corresponding to one standard deviation.

Table 6

Signal sequence	dAb pre-induction basal expression, $g.L^{-1}$ (+/- sd or range)
OmpASS	0.30 ± 0.15
YceISS	0.64 ± 0.06
IvySS	0.061 ± 0.006

The strain using the *OmpASS* (Δ), demonstrated a typical phase of rapid growth followed by cell autolysis as evidenced by a simultaneous decrease in OD_{600nm} (Figure 2A) and increase in extracellular dsDNA (Figure 2B). This coincided with rapid formation of dAb with release into the extracellular environment/supernatant (Figures 2C & D). The total dAb formed is summarised in Figure 2E, with nearly all occurring extracellularly by the end of the fermentation (Figure 2F). At the point of induction a significant amount (0.30 $g.L^{-1}$) of dAb had been already expressed in the cells (Figure 2C & legend). This was accompanied by the formation of a highly viscous, shear thinning broth by the start of the induction phase (Figures 2G & H). The broth viscosity continued to increase for 24 h during the post-induction phase (Figure 2G) with a parallel increase in extracellular dsDNA levels.

Similar trends to the use of *OmpASS* culture are observed for the use of *YceISS* (□). Here the product expression level at induction (0.64 $g.L^{-1}$) is greater than for strains using *OmpASS*. This is accompanied by a greater broth viscosity at induction and subsequently, reduced cell growth and slightly lower product levels either as total or as extracellular dAb.

In contrast, the use of *IvySS* (◊) led to low dAb expression levels of 0.061 $g.L^{-1}$ at the time of induction and a low viscosity broth. This is followed by a viscosity increase throughout the fermentation progression in parallel with an increased dsDNA release (Figure 2B). Cell growth continues for a longer period before the onset of lysis at ~ 72 h i.e. compared with ~12 h observed for the use of *OmpASS* and *YceISS*. The resultant effect is a slower rate of dAb expression and delayed release of dAb to the extracellular space

(Figures 2D & F). Overall it appears that the use of *IvySS* compared with *OmpASS* and *YceISS* offers tighter control of dAb formation prior to induction.

Example 3

An amino acid sequence alignment between OmpASS, YceISS and IvySS (Figure 3A) and evaluation of their codon adaptation index (CAI) (Figure 3B & Table 3) identified a cassette of eight amino acids (Ivy'SS) at the N-terminus of IvySS that was significantly different from OmpASS and YceISS in terms of amino acid sequence, but also contained a large proportion of rare codons (Table 3).

Figure 3 shows signal sequences amino acid sequence alignments and evaluation of their codon adaptation indices. (A) Amino acid sequence alignments for *YceISS* (SEQ ID NO: 35) and *IvySS* (SEQ ID NO: 34) are referenced against the *OmpASS* (SEQ ID NO: 33). Conserved amino acids are highlighted. (B) Adaptiveness of each codon position was calculated as a ratio of that codon frequency against the most abundant synonymous codon with reference to the *E. coli* codon usage table derived from the Kazusa *E. coli* codon usage database. Amino acid sequences in 'B' are aligned from the first methionine after the *Ivy'SS* cassette.

It was hypothesized that the presence of this eight-codon cassette or the resulting amino acids for *IvySS* might affect the level and the rate of dAb expression before induction (i.e. the basal level). To investigate the impact of the identified *Ivy'SS* cassette in facilitating dAb formation during high cell density fed-batch fermentation, two modified signal sequences were developed; *IvyTruncSS*, a signal sequence based on that for *IvySS* with *Ivy'SS* removed; and also *Ivy'OmpASS*, which is the product of the fusion of the codons for *Ivy'SS* to the N-terminus of *OmpASS*. Additionally, two codon optimised *IvySS* signal sequences, *IvyOpt1SS* and *IvyOpt2SS*, were created (Figure 3B, and Materials and Methods for details) to explore if the rate of expression of the dAb can be increased without changing the basal dAb expression level and the broth viscosity. The resultant CAI values for the four modified *Ivy* related signal sequences are given in Table 4.

Example 4

The growth of *E. coli* W3110 cultures expressing dAb with the modified signal sequence is described in Figure 4. All the modified signal sequences presented in Figure 4 were efficiently cleaved resulting in a correctly folded dAb molecule.

Figure 4 shows post-induction fermentation profiles for high cell density *E. coli* W3110 fed-batch cultures. N-terminal signal sequence and basal (pre-induction) expression level: (Δ), *IvyTruncSS*, 0.90 $g.L^{-1}$, (□), *Ivy'OmpASS*, 0.086 $g.L^{-1}$, (◊), *IvyOpt1SS*,

0.16 g.L^{-1} and (♦), *IvyOpt2SS*, 0.089 g.L^{-1}. See Figure 2 for details. Results were taken from single fermentation samples (m = 3).

Table 7

Signal sequence	dAb pre-induction basal expression, g.L^{-1} (+/- sd or range)
IvyTruncSS	0.90 ± 0.09
Ivy'OmpASS	0.086 ± 0.008
IvyOpt1SS	0.16 ± 0.01
IvyOpt2SS	0.089 ± 0.008

The use of the *IvyTruncSS* resulted in earlier cessation of growth, greater dsDNA release and lower dAb formation than observed for the use of *IvySS* (Figure 2). The level of basal expression of the dAb at the time of induction is higher than for the use of *IvySS* and the viscosity of the both just prior to and after induction is significantly higher. Hence the presence of *Ivy'SS* appears to be critical to reducing the early expression prior to induction and hence the resultant high viscosity levels (Figure 4G). The increased rate of expression observed (Figure 4E) is consistent with the removal of a sequence containing a large proportion of rare codons. The use of the *Ivy'OmpASS* resulted in growth, dsDNA release, dAb formation and location and viscosity profiles similar to those observed when using the *IvySS* (Figure 2) confirming the importance of *Ivy'SS* in reducing basal dAb expression. Again a low viscosity is observed for *Ivy'OmpASS* at the start of the induction phase and the subsequent viscosity rise is attributed to the presence of extracellular dsDNA. Similarly, as with *IvySS*, extended fermentation times were needed to yield significant expression levels of dAb and release to the extracellular environment. The use of *IvyOpt1SS* and *IvyOpt2SS* resulted in low basal expression of the dAb at the point of induction (Figure 4 legend). The result is a low viscosity broth (Figure 4G). As expected, the reduced incidence of rare codons in the optimised signal sequences leads to an increased rate of dAb formation (Figure 4E). Again, as with the use of *IvySS*, the rise in viscosity of the broth post-induction is attributable to dsDNA release. *IvyOpt2SS* appears to yield a lower basal expression, a higher dAb titre and a lower viscosity broth than for *IvyOpt1SS*.

Example 5

The fermenter productivity as measured for the total titre post-induction, is compared in Figure 5A for the range of signal sequences studied. The low values of dAb for strains using *IvySS* and *Ivy'OmpASS* are evidently due to the extended times (~150 h) needed for product formation and release. Strains using *IvyOpt1SS*, *IvyOpt2SS* and *IvyTruncSS* all yield maximum dAb concentration within 24 h of induction and high fermenter productivity levels. Mass spectrometry analysis (not shown here) suggested the

signal sequence was completely removed from the dAb for all of the constructs studied in Figure 5. The clarification of the final broths using USD centrifugation is summarised in Fig 5B. The conditions used are representative of industrial scale disc stack or solid bowl continuous flow centrifuges operating at low end of this flow rate capacity and fitted with a high shear stress feed zone. A no shear stress feed, as for a bench top centrifuge, is mimicked as a comparative control.

Figure 5 (A) shows overall productivity calculated as the mass of extracellular dAb, per litre of broth per total post-induction time; and (B) shows centrifugation performance of *E. coli* harvest cultures expressing a dAb with different N-terminal signal sequences; OmpASS (48h post-induction), *YceISS* (48h), *IvyTruncSS* (55h), *Ivy'OmpA* (153h) and *Ivy* codon optimised signal sequences *IvyOpt1* (48h) and *IvyOpt2* (55h). Cell broths were exposed to conditions of: (■), no shear; (□), high shear stress (maximum energy dissipation rate (ε) of 0.53 $x10^6$ $W.kg^{-1}$ for 20 s), and then processed by USD centrifugation (V = 2.0 mL, t = 420 s, Σ = 0.164 m^2, (see text), $V/t\Sigma = 2.9 \times 10^{-8}$ $m.s^{-1}$,). Results were taken from replicated fermenters (*n* = 3) for *OmpASS* and *YceISS* or single fermenter samples for remaining signal sequences. Sample characterization for (B) was in triplicate (*m* = 3). Results shown as mean +/- SD.

The high viscosities of the broths obtained using *OmpASS* and *YceISS* resulted in a poorly clarified supernatant with ~50% and ~80% of solids still in the supernatant respectively. The remaining broths are all clarified to >10 fold greater extent (~1-4% remaining solids) so that the differences in performance are less obvious. The medium viscosity broth obtained when using the *IvyTruncSS* (Fig 4G) resulted in a clarified broth at the high end of this range (~4%). The remaining broths were all clarified to a greater extent reflecting the even lower viscosities of the final broths. In nearly all cases, the effect of high shear stress resulted in 5-30% improvement in clarification indicating a probable reduction of viscosity e.g. due to break up of fluid structures (such as polymer bridges). Several strains result in both high fermenter productivity and good centrifugal clarification. Strains using *IvyOpt2SS* followed by *IvyOpt1SS* provide the most promising choices based on this study. The use of IvyTruncSS also does perform well, but the indication is that clarification by centrifugation is more challenging.

Summary of Examples

Two signal sequences, *IvySS* and *YceISS* have been identified, which enable the secretion of a heterologous protein in *E. coli* periplasmic space. A strain using *YceISS* was shown to perform in a 1 L fed-batch, stirred-tank fermenter in a comparable way with the use of *OmpASS* (Figure 2). The use of the *IvySS* resulted in considerably lower broth

viscosities compared to those obtained with the use of *OmpASS* and *YcelSS* albeit with slower production rates (Figure 2 & Table 3) and lower overall productivities (Figure 5A). We discuss firstly the reason for the formation of high viscosity broths with the media and fermentation strategy used here and secondly the development of signal sequences based on *IvySS*.

The initial studies using *OmpASS*, *YcelSS* and *IvySS* suggest that a high basal (i.e. pre-induction) expression of dAb results in the formation of a highly viscous fermentation broth at the start of induction (Figure 2). Such high viscosities are continued throughout the remainder of the fermentation with any further increase probably attributable to DNA release with increased cell autolysis. This relationship between basal expression and viscosity is supported by observations using a range of other signal sequences (Figure 4) and is summarised in Table 5. Preliminary studies, not shown here, demonstrated a high viscosity broth (K ~ 1.1 Ns^nm^{-2}) for *IvySS* when induction was carried out during rather than after the end of the exponential growth phase mimicking a high basal expression.

The high viscosities observed by the time of induction can probably be attributed to formation of extracellular polymeric substances (EPS) during fermentation, these being a key component of microbial biofilms. Factors such as growth phase, media composition, temperature, oxygen limitation, nitrogen and cation deficiency have been shown to control EPS biosynthesis. For other microbial systems, exponential growth itself has been linked to the formation of EPS and biofilm. Acetate has also been linked to EPS formation. Biofilm and EPS formation in general has been shown to lead to reduction of growth rate. Observations in this study suggest it is possible that the expression of dAb combined with the effect of environmental conditions pre-induction might promote EPS formation.

For the fermentations studied here the control strategies for the DO are such that oxygen limitation (DO ~ 0%), causing hypoxic conditions, occurs in the exponential growth phase before induction. Both hypoxia and exponential growth are linked to formation of fermentative metabolism by-products and overflow metabolism products respectively, such as acetate. Such conditions, combined with the presence of high basal levels of dAb are possibly leading to EPS formation. Subsequent to induction, the conditions are such that any acetate formed is likely to have been consumed before the start of the nutrient feed. From this point on, the controlled constant nutrient feed rate will mean that cells are in a linear or stationary phase of growth and the DO control is efficiently maintained at 30%; *i.e.* there is unlikely to be any significant levels of acetate formation present and hence no further EPS formation despite the high levels of dAb. Hence, the control of fermentation conditions prior to induction may be used to avoid acetate formation. Alternatively, media adjustment can diminish biofilm formation e.g. reduced presence of soy or the use of

glucose rather than glycerol (as used in this work) as a carbon source. In this study, the fermentations were carried out using a soytone containing medium; the disadvantages of a high viscosity broth when using *OmpASS* to produce the same dAb have been overcome through the use of soy-free fermentation media. Basal expression (and hence for the system studied here, EPS formation) might be reduced by use of a more tightly controlled expression system but this may generate other process related challenges. Another potential route to eliminate EPS formation is by the use of a biofilm deficient *E. coli* strain. Future work will seek to confirm the impact of EPS leading to the high viscosities observed in this study. The focus here is on how selected signal sequences may be improved to achieve low viscosity fermentation using the existing media and fermentation operating conditions.

Two aspects of the make-up of *IvySS* appear to relate to its performance in determining the basal expression and the post-induction productivity of the dAb. Firstly the presence of an eight codon cassette (*Ivy'SS*) at the start of *IvySS* leads to tight control of protein expression before induction, i.e. low basal expression. Secondly a low codon adaptation index (CAI) appears to lead to a low rate of expression of the protein. The effect of choice of signal sequence, including modified Ivy signal sequences to retain low basal expression while increasing productivity, is summarized in Table 5. When *Ivy'SS* was present at the N-terminus of the signal sequence, the level of basal expression prior to induction of dAb expression was low and a broth of low viscosity was obtained. The low basal levels achieved with *IvyOpt1SS* and *IvyOpt2SS* (Figure 4) suggest that it is the amino acid sequence, and not the presence of rare codons in the gene sequence which determines the basal expression level.

The initial productivity is possibly related to the CAI which in turn is linked to the rate of translation elongation and dAb formation. The presence of rare codons at the 5' terminus of mRNA has been hypothesized to lead to slow elongation of the nascent polypeptide during translation to reduced ribosome traffic jams, this leading to lower initial cell productivity. From Table 5, a low CAI to sequence length value led to a low initial productivity e.g. as seen for *IvySS* and *Ivy'OmpASS*, while for all other signal sequences there is a high CAI per codon number value leading to high initial productivity. Ideally this analysis should be weighted towards the N terminal end of the construct. This is particularly when rare codons are present for the two strains giving low initial productivity as for the ones using *IvySS* and *Ivy'OmpASS*. The increase in the initial productivity of dAb for *IvyOpt1SS* and *IvyOpt2SS* compared with that obtained using *IvySS* could be due to either the modified codon sequence or the removal of rare codons.

IvyOpt2SS is designed by optimisation based on a reference set of optimal codons frequently represented in genes that are highly and continuously expressed during exponential growth while *IvyOpt1SS* is designed based on expression of all *E.coli* genes. The former leads to higher CAI per codon number (Table 5) and this might lead to the greater overall productivity noted (Figure 5A). Future work will explore further the potential for such optimisation to further improve the overall expression level while still preventing significant basal expression leading to high viscosity broths.

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Other Sequences

SEQ ID NO: 53: Amino acid sequence of DOM0101

EVQLLESGGGLVQPGGSLRLSCAASGFTFAHETMVWVRQAPGKGLEWVSHIPPDGQD PFYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYHCALLPKRGPWFDYWGQGTLV TVSS

SEQ ID NO: 54: DNA sequence of DOM0101 – (no signal sequence)

GAAGTACAACTGCTGGAGAGCGGTGGCGGCCTGGTTCAACCGGGTGGTTCCCTGC GCCTGTCCTGTGCGGCATCTGGTTTCACCTTCGCACACGAAACCATGGTGTGGGTT CGCCAAGCTCCGGGCAAAGGCCTGGAATGGGTAAGCCACATTCCTCCAGATGGCCA GGACCCATTCTATGCGGATTCCGTTAAGGGTCGCTTTACCATTTCTCGTGATAACTC CAAAAACACCCTGTACCTGCAGATGAACTCCCTGCGCGCCGAGGATACTGCGGTGT

ACCATTGTGCGCTGCTGCCTAAACGTGGCCCGTGGTTCGATTACTGGGGTCAGGGT
ACTCTGGTCACCGTAAGCAGC