METHODS AND COMPOSITIONS FOR PREVENTING NORLEUCINE MISINCORPORATION INTO PROTEINS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/777,700, filed on 12 March 2013, and U.S. Provisional Application No. 61/703,142, filed on 19 September 2012, both of which are incorporated by reference herein in their entireties.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 17, 2013, is named P4967R1_WO_SequenceListing.txt and is 45,847 bytes in size.

FIELD OF THE INVENTION

The present invention relates to methods and compositions for preventing misincorporation of norleucine into proteins during recombinant protein production in bacteria. The present invention also provides microorganism host cells and nucleic acid molecules for use with the methods and compositions provided herein.

BACKGROUND

Norleucine, an analog of the amino acid methionine, can be misincorporated into proteins in place of methionine residues. When expressed in *Escherichia coli* (*E. coli*), many heterologous proteins have norleucine mistakenly incorporated in places where methionine residues should appear. The misincorporation of norleucine in proteins, in particular in heterologous proteins produced by recombinant means, is generally considered undesirable due, in part, to the resulting production of altered proteins having undesirable characteristics.

Misincorporation of norleucine at methionine positions during recombinant protein production in *E. coli* has been observed for over 50 years. (See, *e.g.*, Munier and Cohen (1959) Biochim Biophys Acta 31:378-391; Cohen and Munier (1956) Biochim Biophys Acta

21:592-593; Cohen and Munier (1959) Biochim Biophys Acta 31:347-356; and Cowie *et al.*, (1959) Biochim Biophys Acta 34:39-46.) For example, approximately 14% of methionine residues in methionyl bovine somatotropin (MBS) exhibited norleucine misincorporation during recombinant production of this protein in *E. coli,* and approximately 6% of the methionine residues in native *E. coli* proteins were also substituted with norleucine. (See Bogosian *et al.*, (1989) J Biol Chem 264:531-9.) In another example, production of interleukin-2 in a minimal medium *E. coli* fermentation resulted in approximately 19% of the methionine residues in the recombinant protein were substituted with norleucine. (See Tsai *et al.*, (1988) Biochem Biophys Res Commun 156:733-739.) Other studies showed that norleucine residue misincorporation into protein can occur both at internal methionine residues and at the amino terminal methionine residue. (See Brown (1973) Biochim Biophys Acta 294:527-529; and Barker and Bruton (1979) J Mol Biol 133:217-231.)

Norleucine competes with methionine for incorporation into proteins due to the promiscuous nature of the enzyme methionyl tRNA synthetase (MetG). (See Trupin *et al.*, (1966) Biochem Biophys Res Commun 24:50-55; and Fersht and Dingwall (1979) Biochemistry 18:1250-1256.) Kinetic studies with *E. coli* MetG enzyme showed that acylation of MetG is approximately 4-fold higher with methionine compared to that with norleucine. (See van Hest *et al.*, (2000) Am Chem Soc 122:1282-1288.) Due to the relaxed substrate specificity of MetG, norleucine can substitute for methionine in the acylation reaction, resulting in misincorporation of norleucine into proteins in place of methionine.

Misincorporation of norleucine residues for methionine residues in recombinant protein production is generally considered undesirable. Recombinant proteins or polypeptides containing misincorporated norleucine residues may exhibit altered structural and functional features, such as, for example, altered sensitivity to proteolysis, diminished biological activity, or increased immunogenicity.

Various strategies have been developed to reduce or prevent norleucine misincorporation during recombinant protein production. For example, supplementing cell culture medium with methionine during the fermentation process (by continuous or bolus feed/addition of methionine) has been used to ensure that excess methionine is available to the cells, thus reducing the probability of an incorrect charging of the methionyl tRNA with norleucine.

(See, *e.g.*, United States Patent No. 5,599,690.) While continuous or bolus feed/addition of methionine reduced the extent of norleucine misincorporation in recombinant proteins, the operational complexity and cost of the fermentation process may increase. Furthermore, continuous or bolus feed/addition of methionine during fermentation may lead to undesirable dilution of the fermentor contents, resulting in lower cell densities and lower product yields.

Deleting genes involved in the norleucine biosynthetic pathway such as, for example, deleting genes of the leucine operon (*leuA*, *leuB*, *leuC*, and *leuD*) or deleting transaminase encoding genes such as *ilvE or tyrB,* has also been used to reduce norleucine misincorporation in proteins. (See Bogosian *et al.*, (1989) J Biol Chem 264:531-539; Tsai *et al.*, (1989) Biochem Biophys Res Commun 156:733-739; and Randhawa *et al.*, (1994) Biochemistry 33:4352-4362.) The deletion of biosynthetic pathway genes to prevent norleucine misincorporation, however, may require addition of other amino acids (such as leucine or isoleucine) to the culture medium during fermentation as many genes involved in norleucine biosynthesis are also involved in biosynthesis of branched chain amino acids. (See Bogosian *et al.*, (1989) J Biol Chem 264:531-539; see Figure 8 of the instant specification.)

Another strategy used to prevent norleucine misincorporation involved co-expression of enzymes which degrade norleucine, including, for example, amino acid dehydrogenases and amino acid oxidases. This approach, however, required overexpression of these enzymes, which may not be desirable during recombinant protein production, and may lead to lower recombinant protein yields. (See *e.g.*, United States Patent Application Publication No. US2007/0009995.) In addition, over expression of these enzymes may result in degradation of other analogous amino acids during the fermentation process. Altering the primary amino acid sequence of the polypeptide to be expressed by substituting methionine codons with other codons was also performed to prevent norleucine misincorporation. (See *e.g.*, United States Patent No. 5,698,418.) Such substitutions, however, may lead to diminished activity or structural changes in the resulting protein, a highly undesirable outcome for recombinant protein production in the biotechnology industry.

As noted above, current methods used to prevent norleucine misincorporation during recombinant protein production in microorganisms are associated with various disadvantages;

therefore, a need exists for novel methods useful for preventing or reducing norleucine misincorporation in to proteins, in particular during recombinant protein production in microorganisms, such as *E. coli*.

The present invention meets this need by providing engineered microorganism host cells effective at preventing norleucine misincorporation during recombinant protein production in microorganisms, such as, for example, bacteria. The present invention provides, *inter alia*, *E. coli* host cells comprising mutated *metA* and *metK* alleles (*i.e.*, altered *metA* and *metK* nucleic acid sequences) which result in methionine production by the microorganism to a degree or extent sufficient to reduce or prevent norleucine misincorporation into proteins and polypeptides. Analysis of recombinant proteins produced utilizing such host cells showed that misincorporation of norleucine residues in place of methionine residues was eliminated. The present invention further demonstrates that fermentation process performance using such *E. coli* host cells, including growth of the host cells and recombinant protein product titers utilizing such *E. coli* host cells, was comparable to that observed in control host cells.

SUMMARY

The present disclosure provides, in part, methods and compositions for preventing or reducing norleucine misincorporation into proteins and polypeptides. The methods and compositions of the present disclosure are useful for preventing or reducing norleucine misincorporation in heterologous (*e.g.*, recombinant) proteins and polypeptides expressed by a microorganism, such as, for example, bacteria (*e.g., E. coli*).

Disclosed is a method for preventing or reducing norleucine misincorporation into a protein or polypeptide, the method comprising expressing the protein or the polypeptide in a microorganism, wherein the microorganism is a mutant microorganism which produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into the protein or polypeptide.

In one aspect, there is provided a method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

In one aspect, there is provided a method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

In one aspect, there is provided a method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and further wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

In some embodiments, the present disclosure provides methods for preventing or reducing norleucine misincorporation into a protein or polypeptide expressed by a microorganism, wherein the microorganism produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into the protein or polypeptide. In some embodiments, the microorganism is a feedback-resistant or feedback-insensitive homoserine succinyltransferase microorganism. In other embodiments, the microorganism is a microorganism comprising a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele.

In some embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetA selected from the group consisting of an arginine to cysteine substitution at amino acid position 27, a glutamine to glutamic acid substitution at amino acid position 64, a tyrosine to cysteine substitution at amino acid position 294, an isoleucine to serine substitution at amino acid position 296, and a proline to leucine substitution at amino acid position 298. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence encoding amino acid substitutions in MetA comprising an isoleucine to serine substitution at amino acid position 296 and a proline to leucine substitution at amino acid position 298. MetA amino acid positions described herein in the instant specification are in reference to wild-type MetA amino acid sequence as shown in Figure 7A and SEQ ID NO:29.

In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

As stated above, the present disclosure provides methods for preventing or reducing norleucine misincorporation into a protein or polypeptide expressed by a microorganism, wherein the microorganism produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into the protein or polypeptide. In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide expressed by a microorganism, the method comprising expressing the protein or polypeptide in the microorganism, wherein the microorganism is a microorganism de-repressed for methionine production. In some embodiments, the microorganism is de-repressed for methionine production due to partial loss-of-function of S-adenosylmethionine synthase. In other embodiments, the present

disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide expressed by a microorganism, the method comprising expressing the protein or polypeptide in the microorganism, wherein the microorganism comprises a mutant *metK* allele. In some embodiments, the mutant *metK* allele results in a partial loss-of-function of MetK.

In some embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetK comprising a valine to glutamic acid substitution at amino acid position 185. In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence comprising a deletion of the cytosine base at nucleic acid residue position 1132 of the *metK* allele. MetK amino acid positions described herein in the instant specification are in reference to wild-type MetK amino acid sequence as shown in Figure 8A and SEQ ID NO:30. *metK* nucleic acid positions described herein in the instant specification are in reference to wild-type *metK* nucleic acid sequence as shown in Figure 8B and SEQ ID NO:32.

In some embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a *metK* allele. In some embodiments, the mutant *metA* allele

comprises a nucleic acid sequence encoding a tyrosine to cysteine substitution at amino acid position 294 of MetA and the mutant *metK* allele comprises a nucleic acid sequence encoding a valine to glutamic acid substitution at amino acid position 185 of MetK. In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding a tyrosine to cysteine substitution at amino acid position 294 of MetA, and wherein the mutant *metK* allele comprises a nucleic acid sequence comprising a deletion of the cytosine base at nucleic acid residue position 1132 of the *metK* allele.

In other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, and wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:27. In yet other embodiments, the present disclosure provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:28.

The present disclosure further provides microorganism host cells useful for preventing or reducing norleucine misincorporation into proteins and polypeptides expressed by a microorganism host cell. The present disclosure also provides microorganism host cells for use in the expression of proteins or polypeptides by the microorganism host cell, wherein the expressed proteins or polypeptides are free of norleucine misincorporation. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

Disclosed is a microorganism comprising a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment.

In one aspect, there is provided an *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment.

The present disclosure provides a microorganism (*e.g.*, a microorganism host cell), wherein the microorganism produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into proteins or polypeptides expressed by the microorganism. In some embodiments, the present disclosure provides a microorganism, wherein the microorganism is a feedback-insensitive homoserine succinyltransferase microorganism. In other embodiments, the present disclosure provides a microorganism comprising a mutant *metA* allele. In some embodiments, the present disclosure provides a microorganism comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetA selected from the group consisting of an arginine to cysteine substitution at amino acid position 27, a glutamine to glutamic acid substitution at amino acid position 64, a tyrosine to cysteine substitution at amino acid position 294, an isoleucine to serine substitution at amino acid position 296, and a proline to leucine substitution at amino acid position 298. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

In some embodiments, the present disclosure provides a microorganism comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding more than one amino acid substitution in MetA. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence encoding an isoleucine to serine substitution at amino acid position 296 in MetA and a proline to leucine substitution at amino acid position 298 in MetA. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

In some embodiments, the present disclosure provides a microorganism, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

The present disclosure provides a microorganism (*e.g.*, a microorganism host cell), wherein the microorganism produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into proteins or polypeptides expressed by the microorganism.

In some embodiments, the present disclosure provides a microorganism, wherein the microorganism is a microorganism de-repressed for methionine production. In some embodiments , the microorganism de-repressed for methionine production results from a partial loss-of-function of S-adenosylmethionine synthase. In other embodiments, the present disclosure provides a microorganism comprising a mutant *metK* allele. In some embodiments, the present disclosure provides a microorganism comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetK comprising a valine to glutamic acid substitution at amino acid position 185. In other embodiments, the present disclosure provides a microorganism comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence comprising a deletion of the cytosine base at nucleic acid residue position 1132 in the *metK* allele. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

In some embodiments, the present disclosure provides a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

The present disclosure also provides microorganism host cells comprising various combinations of mutant *metA* alleles and a mutant *metK* alleles. In some embodiments, the present disclosure provides a microorganism comprising a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetA comprising a tyrosine to cysteine substitution at amino acid position 294, and wherein the mutant *metK* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetK comprising a valine to glutamic acid substitution at amino acid position 185. In other embodiments, the present disclosure provides a microorganism comprising a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding an amino acid substitution in MetA comprising a tyrosine to cysteine substitution at amino acid position 294, and wherein the mutant *metK* allele comprises a deletion of the nucleic acid cytosine at nucleic acid residue 1132 of the

metK allele. In some embodiments, the present disclosure provides a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and wherein the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:27. In other embodiments, the present disclosure provides a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and wherein the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:28. In some embodiments, the microorganism host cell is a bacterium. In other embodiments, the microorganism host cell is *E. coli*.

The present disclosure also provides isolated nucleic acid molecules for use in the present methods. In some embodiments , the present disclosure provides isolated *metA* nucleic acid molecules (*i.e.*, isolated nucleic acid molecules encoding MetA). In some embodiments, the present disclosure provides an isolated *metA* nucleic acid molecule, wherein the *metA* nucleic acid molecule comprises a nucleic acid sequence encoding an amino acid substitution in MetA selected from the group consisting of an arginine to cysteine substitution in at amino acid position 27, a glutamine to glutamic acid substitution at amino acid position 64, a tyrosine to cysteine substitution at amino acid position 294, an isoleucine to serine substitution at amino acid
position 296, and a proline to leucine substitution at amino acid position 298. In other embodiments, an isolated *metA* nucleic acid molecule provided by the present disclosure comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. The use of these isolated *metA* nucleic acid molecules and sequences thereof for the production of microorganisms for use in preventing or reducing norleucine misincorporation in proteins or polypeptides is specifically provided herein by the present disclosure .

The present disclosure also provides isolated *metK* nucleic acid molecules (*i.e.*, isolated nucleic acid molecules encoding MetK). In some embodiments, the present disclosure provides a *metK* nucleic acid molecule, wherein the *metK* nucleic acid molecule comprises a nucleic acid sequence encoding an amino acid substitution in MetK comprising a valine to glutamic acid substitution at amino acid position 185. In other embodiments, the present

disclosure provides a *metK* nucleic acid molecule, wherein the *metK* nucleic acid molecule comprises a deletion of the nucleic acid cytosine at nucleic acid residue 1132 of the *metK* allele. In other embodiments, a *metK* nucleic acid molecule provided by the present disclosure comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. The use of these isolated *metK* nucleic acid molecules and sequences thereof for the production of microorganisms for use in preventing or reducing norleucine misincorporation in proteins or polypeptides is specifically provided herein by the present disclosure .

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 46 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 47. In some embodiments, the nucleic acid encoding the amino acid sequence of SEQ ID NO:46 is the nucleic acid sequence of SEQ ID NO:33. In some embodiments, the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:47 is the nucleic acid sequence of SEQ ID NO:34. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:33 and a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:34. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid

sequence of SEQ ID NO: 46 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 47. In some embodiments, the nucleic acid encoding the amino acid sequence of SEQ ID NO:46 is the nucleic acid sequence of SEQ ID NO:33. In some embodiments, the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:47 is the nucleic acid sequence of SEQ ID NO:34. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:33 and a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:34. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments, the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 46 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 47. In some embodiments, the nucleic acid encoding the amino acid sequence of SEQ ID NO:46 is the nucleic acid sequence of SEQ ID NO:33. In some embodiments, the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:47 is the nucleic acid sequence of SEQ ID NO:34. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:33 and a nucleic acid having the nucleic acid sequence corresponding to SEQ ID NO:34. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 48 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 49. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 48 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 49. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 48 and a nucleic acid encoding the amino acid sequence of SEQ ID NO: 49. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 50, a nucleic acid encoding the amino acid sequence of SEQ ID NO: 51, and a nucleic acid encoding the amino acid sequence of SEQ ID NO:52. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 50, a nucleic acid encoding the amino acid sequence of SEQ ID NO: 51, and a nucleic acid encoding the amino acid sequence of SEQ ID NO:52. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

The present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment. In some embodiments, the present disclosure provides a microorganism comprising a nucleic acid comprising a mutant *metA* allele and a mutant *metK* allele, wherein the microorganism further comprises a nucleic acid encoding the amino acid sequence of SEQ ID NO: 50, a nucleic acid encoding the amino acid sequence of SEQ ID NO: 51, and a nucleic acid encoding the amino acid sequence of SEQ ID NO:52. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and the mutant *metK* allele comprises a nucleic acid

sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments , the microorganism is a bacteria, *e.g., E. coli*.

Disclosed is a method for producing a protein or a polypeptide in a bacteria host cell, wherein the protein or the polypeptide is free of norleucine misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the protein or the polypeptide under culture conditions suitable to allow for expression of the protein or the polypeptide, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing a protein or a polypeptide free of norleucine misincorporation.

The present disclosure further provides a method for producing in a bacteria host cell a protein or a polypeptide free of norleucine misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the protein or the polypeptide, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing a protein or polypeptide free of norleucine misincorporation. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

The present disclosure also provides a method for producing an antibody or an antibody fragment in a bacteria host cell, wherein the antibody or the antibody fragment is free of norleucine misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the antibody or the antibody fragment, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing an antibody or an antibody fragment free of norleucine misincorporation. In some embodiments , the method for producing an antibody or an antibody fragment in a bacteria host cell free of norleucine misincorporation according to the present disclosure comprises expressing in the bacteria host cell a nucleic acid encoding an antibody heavy chain polypeptide and a nucleic acid encoding an antibody light chain polypeptide. In some embodiments , the antibody heavy chain polypeptide is an antibody Fab fragment heavy chain polypeptide, and the antibody light chain polypeptide is an antibody Fab fragment light chain polypeptide. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

In one aspect, there is provided a method for producing a protein or a polypeptide in an *E. coli* host cell, wherein the protein or the polypeptide is free of norleucine misincorporation, the method comprising expressing in the *E. coli* host cell a nucleic acid encoding the protein or the polypeptide under culture conditions suitable to allow for expression of the protein or the polypepetide, wherein the *E. coli* host cell comprises a mutant *metA* allele, wherein the mutant *metA* allele encodes for MetA having an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

In one aspect, there is provided a method for producing a protein or a polypeptide in an *E. coli* host cell, wherein the protein or the polypeptide is free of norleucine misincorporation, the method comprising expressing in the *E. coli* host cell a nucleic acid encoding the protein or the polypeptide under culture conditions suitable to allow for expression of the protein or the polypepetide, wherein the *E. coli* host cell comprises a mutant *metK* allele, wherein the mutant *metK* allele encodes for MetK having an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

In some embodiments, the present disclosure provides a method for producing an anti-VEGF antibody or an anti-VEGF antibody fragment in a bacterial host cell, wherein the anti-VEGF

antibody or the anti-VEGF antibody fragment is free of norleucine misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing an anti-VEGF antibody or an anti-VEGF antibody fragment free of norleucine misincorporation. In some embodiments, the method comprises expressing in the bacteria host cell a nucleic acid encoding an anti-VEGF antibody heavy chain polypeptide or an anti-VEGF antibody fragment heavy chain polypeptide or fragment thereof and a nucleic acid encoding an anti-VEGF antibody light chain polypeptide or an anti-VEGF antibody fragment light chain polypeptide or fragment thereof. In some embodiments , the anti-VEGF antibody heavy chain and the anti-VEGF antibody light chain are full-length heavy chain and light chain anti-VEGF antibody polypeptides. In other embodiments , the anti-VEGF antibody heavy chain is an antibody Fab fragment heavy chain polypeptide, and the anti-VEGF antibody light chain is an antibody Fab fragment light chain polypeptide. In some embodiments, the anti-VEGF antibody heavy chain comprises the amino acid sequence of SEQ ID NO:47 and the anti-VEGF antibody light chain comprises the amino acid sequence of SEQ ID NO:46. In some embodiments, the nucleic acid encoding the amino acid sequence of SEQ ID NO:47 is the nucleic acid sequence of SEQ ID NO:34. In some embodiments, the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:46 is the nucleic acid sequence of SEQ ID NO:33. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

Disclosed is an anti-VEGF antibody or anti-VEGF antibody fragment produced according to the methods as described herein.

The disclosure further provides an anti-VEGF antibody or anti-VEGF antibody fragment produced by any of the methods described herein, wherein the anti-VEGF antibody or anti-VEGF antibody fragment is free of norleucine misincorporation.

In some embodiments, the present disclosure provides a method for producing an anti-Factor D antibody or an anti-Factor D antibody fragment in a bacterial host cell, wherein the anti-Factor D antibody or the anti-Factor D antibody fragment is free of norleucine

misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the anti-Factor D antibody or the anti-Factor D antibody fragment, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing an anti-Factor D antibody or an anti-Factor D antibody fragment free of norleucine misincorporation. In some embodiments, the method comprises expressing in the bacteria host cell a nucleic acid encoding an anti-Factor D antibody heavy chain polypeptide or an anti-Factor antibody fragment heavy chain polypeptide or fragment thereof and a nucleic acid encoding an anti-Factor D antibody light chain polypeptide or an anti-Factor D antibody fragment light chain polypeptide or fragment thereof. In some embodiments , the anti-Factor D antibody heavy chain and the anti-Factor D antibody light chain are full-length heavy chain and light chain anti-Factor D antibody polypeptides. In other embodiments, the anti-Factor D antibody heavy chain is an antibody Fab fragment heavy chain polypeptide, and the anti-Factor D antibody light chain is an antibody Fab fragment light chain polypeptide. In some embodiments, the anti-Factor D antibody heavy chain comprises the amino acid sequence of SEQ ID NO:49 and the anti-Factor D antibody light chain comprises the amino acid sequence of SEQ ID NO:48. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

Disclosed is an anti-Factor D antibody or anti-Factor D antibody fragment produced according to the methods as described herein.

The disclosure further provides an anti-Factor D antibody or anti-Factor D antibody fragment produced by any of the methods described herein, wherein the anti-Factor D antibody or anti-Factor D antibody fragment is free of norleucine misincorporation.

In some embodiments, the present disclosure provides a method for producing an anti-MET antibody or an anti-MET antibody fragment in a bacterial host cell, wherein the anti-MET antibody or the anti-MET antibody fragment is free of norleucine misincorporation, the method comprising expressing in the bacteria host cell a nucleic acid encoding the anti-MET antibody or the anti-MET antibody fragment, wherein the bacteria host cell comprises a mutant *metA* allele, a mutant *metK* allele, or a mutant *metA* allele and a mutant *metK* allele, thereby producing an anti-MET antibody or an anti-MET antibody fragment free of

norleucine misincorporation. In some embodiments, the method comprises expressing in the bacteria host cell a nucleic acid encoding an anti-MET antibody heavy chain polypeptide or an anti-MET antibody fragment heavy chain polypeptide or fragment thereof and a nucleic acid encoding an anti-MET antibody light chain polypeptide or an anti-MET antibody fragment light chain polypeptide or fragment thereof. In some embodiments , the anti-MET antibody heavy chain and the anti-MET antibody light chain are full-length heavy chain and light chain anti-MET antibody polypeptides. In other embodiments , the anti-MET antibody heavy chain is an antibody Fab fragment heavy chain polypeptide, and the anti-MET antibody light chain is an antibody Fab fragment light chain polypeptide. In some embodiments, the anti-MET antibody heavy chain comprises the amino acid sequence of SEQ ID NO:51, the anti-MET antibody heavy chain fragment comprises the amino acid sequence of SEQ ID NO:52, and the anti-MET antibody light chain comprises the amino acid sequence of SEQ ID NO:50. In some embodiments, the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26. In some embodiments, the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

Disclosed is an anti-MET antibody or anti-MET antibody fragment produced according to the methods as described herein.

The disclosure further provides an anti-MET antibody or anti-MET antibody fragment produced by any of the methods described herein, wherein the anti-MET antibody or anti-MET antibody fragment is free of norleucine misincorporation.

In various embodiments , a mutant microorganism comprising any one or more of the nucleic acid sequences provided by the present disclosure is bacteria; in other embodiments , the microorganism is *E. coli*. The present disclosure specifically provides for the use of a mutant microorganism described herein for the production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein the misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced, substantially reduced, substantially eliminated, or prevented.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleic acid sequence of *metA*(R27C) corresponding to SED ID NO:23.

Figure 2 shows the nucleic acid sequence of *metA*(Y294C) corresponding to SEQ ID NO:24.

Figure 3 shows the nucleic acid sequence of *metA*(I296S/P298L) corresponding to SEQ ID NO:25.

Figure 4 shows the nucleic acid sequence of *metA*(Q64E) corresponding to SEQ ID NO:26.

Figure 5 shows the nucleic acid sequence of *metK*(V185E) corresponding to SEQ ID NO:27.

Figure 6 shows the nucleic acid sequence of *metK*(c1132del) corresponding to SEQ ID NO:28.

Figures 7A and 7B show the amino acid sequence and nucleic acid sequence of wild-type MetA corresponding to SEQ ID NO:29 and SEQ ID NO:31, respectively.

Figures 8A and 8B show the amino acid sequence and nucleic acid sequence of wild-type MetK corresponding to SEQ ID NO:30 and SEQ ID NO:32, respectively.

Figure 9 shows the structure of norleucine and norleucine analogues. Norleucine is a structural analogue of methionine, where the sulfur (S) atom is replaced by a methylene group (*i.e.*, $-CH_2$).

Figure 10 shows a schematic of the norleucine biosynthetic pathway in *E. coli*. Dotted arrows indicate that multiple steps are involved. Pyruvate is converted to α-ketocaproate by three passes through the keto acid chain elongation process catalyzed by enzymes encoded by the leucine operon *leuABCD*. The intermediate α-ketocaproate is transaminated to norleucine by transaminases IlvE or TyrB.

Figure 11 shows methionine biosynthesis and regulation in *E. coli*. Dotted arrows indicate feedback inhibition and open arrows indicate repression. Methionine and S-adenosylmethionine (SAM) are feedback inhibitors of the enzyme MetA. The repressor MetJ and its co-repressor SAM inhibit the transcription of enzymes in the methionine regulon.

Figures 12A, 12B, and 12C set forth growth trends, as measured by OD_{550} (Figure 12A) and iOD_{550} (Figure 12B) of 10 L *E. coli* fermentations. The control host (60E4) fermentation was executed with a continuous methionine (■) or continuous water feed (□) (Figure 12A). The 60E4 *metA*(Y294C) host fermentation was performed with continuous water feed (Δ) or no feed (▲) (Figure 12A). Figure 12C shows growth trends for control host 60E4 with no feed (squares), for control host 60E4 with methionine feed (circles), and host 60E4 *metA*(Y294C) with no feed (triangles). Fermentations using all other mutants were performed with continuous water feed.

Figures 13A and 13B show extracellular (Figure 13A) and intracellular (Figure 13B) methionine levels for the control host (□) and 60E4 *metA*(Y294C) (Δ) host cell fermentations performed with continuous water feed. Phosphate levels in the extracellular medium are also shown in dotted line plots (Figure 13A) and (Figure 13B) for control host cell (□) and 60E4 *metA* host cell (Y294C) (Δ) fermentations performed with continuous water feed.

Figure 14A and 14B show extracellular (Figure 14A) and intracellular (Figure 14B) methionine levels for mutant host cell strains of the present invention. Extracellular and intracellular methionine levels are also shown for two control host cell fermentations performed with continuous methionine (■) or continuous water feed (□), respectively.

Figure 15 shows extracellular phosphate levels during fermentations.

Figures 16A and 16B show end of run titer (Figure 16A) and time course titer (Figure 16B) of *E. coli* host cell fermentations. The control host cell (60E4) fermentation was performed with a continuous methionine (■) or continuous water feed (□). The 60E4 *metA* host cell (Y294C) fermentation was performed with a continuous water feed (Δ) or no feed (▲). Fermentations using all other mutant host cells were performed with continuous water feed.

Figures 17A and 17B set forth results of western blots performed on whole cell broth samples obtained during the 60E4 host cell (control host cell) and the 60E4 *metA* host cell (Y294C) fermentations, respectively.

Figures 18A and 18B show the nucleic acid sequences of an anti-vascular endothelial growth factor (anti-VEGF) antibody Fab fragment light chain and heavy chain corresponding to SEQ ID NO:33 and SEQ ID NO:34, respectively.

Figures 19A and 19B set forth growth trends of 10 L *E. coli* fermentations, as measured by OD_{550}. The control hosts (66F8 or 64B4) fermentation processes AF2 or AF3, respectively, were executed with no feed (squares) or a continuous methionine feed (circles). The 66F8 *metA*(Y294C) and the 64B4 *metA*(Y294C) host fermentations were performed with no feed (triangles).

Figures 20A, 20B, and 20C set forth recombinant protein product yields using host strains 60E4 (control host) and 60E4 *metA*(Y294C), 66F8 (control host) and 66F8 *metA*(Y294C), and 64B4 (control host) and 64B4 *metA*(Y294C), respectively.

Figures 21A and 21B show the amino acid sequences of an anti-vascular endothelial growth factor (anti-VEGF) antibody Fab fragment light chain and heavy chain corresponding to SEQ ID NO:46 and SEQ ID NO:47, respectively.

Figures 22A and 22B show the amino acid sequences of an anti-Factor D antibody Fab fragment light chain and heavy chain corresponding to SEQ ID NO:48 and SEQ ID NO:49, respectively.

Figures 23A, 23B, and 23C show the amino acid sequences of an anti-MET antibody light chain (SEQ ID NO:50), heavy chain (SEQ ID NO:51), and heavy chain fragment (SEQ ID NO:52).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides, *inter alia*, methods and compositions for preventing misincorporation of norleucine into proteins and polypeptides, in particular during recombinant protein production in microorganisms. The present invention also provides microorganism host cells and nucleic acid molecules for use in the methods of the invention.

General Methods

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology (including recombinant techniques), microbiology, biochemistry, and immunology, which are known and available to one of skill in the art. Such techniques are described in the literature, such as, *Molecular Cloning: A laboratory Manual*, third edition (Sambrook et al., 2001) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (P. Herdewijn, ed., 2004); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction* (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); and *Short Protocols in Molecular Biology* (Wiley and Sons, 1999). Expression of antibody fragments and polypeptides in bacteria are described in, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.)

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which the invention belongs.

Definitions

The terms "heterologous protein" or "heterologous polypeptide" refer to a protein or a polypeptide not naturally synthesized or produced by a cell or organism (*e.g.*, a microorganism) of interest. For example, an *E. coli* cell may produce a human protein or a human polypeptide, and a human protein or a human polypeptide so produced is a heterologous protein or a heterologous polypeptide. Of particular interest in the context of the present invention are those heterologous proteins or heterologous polypeptides comprising methionine. A heterologous protein or a heterologous polypeptide as used herein also refers to a recombinant protein or a recombinant polypeptide.

The term "norleucine misincorporation" refers to the incorporation of a norleucine residue in a protein or polypeptide for which a methionine residue is encoded by the corresponding nucleic acid encoding the protein or polypeptide.

The terms "mutant allele" or "mutated allele" refer to an allele having a nucleic acid sequence which is different from or altered from the nucleic acid sequence of the wild-type allele (*i.e.*, as naturally found within the cell or microorganism of interest).

The terms "mutant microorganism" or "mutated microorganism" refer to a microorganism which contains one or more mutant alleles or mutated alleles.

The phrase "substantially reduced" or "substantially different," as used herein, refers to a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (*e.g.*, norleucine content in a protein or polypeptide).

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extra-chromosomally or at a chromosomal location that is different from its natural chromosomal location.

"Isolated *metA* nucleic acid molecule" or "isolated *metK* nucleic acid molecule" refers to one or more nucleic acid molecules encoding MetA or MetK, respectively, including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell. "Isolated *metA* nucleic acid molecule" or "isolated *metK* nucleic acid molecule" also refers to a mutant *metA* allele or a mutant *metK* allele.

The phrase "protein or polypeptide free of norleucine misincorporation" refers to a protein or polypeptide which contains no detectable levels of norleucine residues.

As used herein, the singular form of "a", "an", and "the" includes the plural references unless indicated otherwise.

Reference to "about" a value or parameter herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (and describes) aspects that are directed to that value or parameter, *per se*. For example, description referring to "about X" includes description of "X."

Methods for Preventing or Reducing Norleucine Misincorporation

The present invention relates, in part, to methods and compositions useful for preventing or reducing norleucine misincorporation into proteins and polypeptides, in particular during recombinant protein production in microorganisms.

Misincorporation of norleucine residues in place of methionine residues during recombinant protein production in *E. coli* has been previously described. One approach currently used to prevent or reduce norleucine misincorporation is by continuous or bolus feed of methionine to the culture medium during the fermentation process. Although this strategy is effective at reducing norleucine misincorporation, several operational disadvantages are associated with continuous or bolus feeding or addition of methionine during the fermentation process. For example, continuous or bolus feed to the culture increases the operational complexity and the overall cost of the fermentation process. In addition, methionine feed leads to undesirable dilution of the fermentation medium resulting in lower cell densities and possibly lower product yields.

To overcome these disadvantages, the present inventors have provided an alternative to continuous or bolus methionine feed in order to prevent or reduce norleucine misincorporation in heterologous protein or polypeptide production. In particular, the present invention provides microorganism (*e.g.*, *E. coli*) host cells engineered to produce methionine

to a degree or extent sufficient to prevent or reduce norleucine misincorporation during recombinant protein production, including recombinant protein production performed at high host cell densities.

E. coli host cell mutants useful for large-scale methionine production were reported previously. (See, *e.g.*, Chattopadhyay *et al.*, (1991) J Gen Microbiol 137:685-691; Nakamori *et al.*, (1999) Appl Microbiol Biotechnol 52:179-185; Usuda and Kurahashi (2005) Appl Environ Microbiol 71:3228-3234; International Patent Application Publication No. WO2005/111202 2005; and United States Patent Application Publication No. US2009/0298135.) Many of these mutant *E. coli* strains contained mutations in three genes associated with the regulation of methionine biosynthesis: *metJ*, *metA,* and *metK*.

Transcriptional regulation of methionine biosynthesis in *E. coli* involves the enzyme MetJ (*metJ* gene product). MetJ is a transcriptional repressor which, when bound to its co-repressor S-adenosylmethionine (SAM), represses the transcription of genes in the methionine regulon, thus regulating the levels of methionine in the cell. (See, *e.g.*, Marincs (2006) *et al.*, Biochem J 396:227-234.) As previously reported, chemical mutagenesis of *E. coli* followed by selection for growth on ethionine (a toxic methionine analogue) led to the isolation of a serine to asparagine mutation at amino acid position 54 (S54N) in MetJ, which resulted in de-repression of methionine biosynthetic enzymes and increased methionine production. (See Nakamori *et al.*, (1999) Appl Microbiol Biotechnol 52:179-185.) A complete disruption of the *metJ* gene also resulted in de-repression of enzymes involved in the methionine biosynthetic pathway and methionine overproduction. (See Usuda and Kurahashi (2005) Appl Environ Microbiol 71:3228-3234.)

Methionine biosynthesis in *E. coli* is also regulated by feedback inhibition (by methionine and SAM) of homoserine succinyltransferase (*metA* gene product), the enzyme involved in the first step of methionine biosynthesis. (See, *e.g.*, Born and Blanchard (1999) Biochemistry 38:14416-14423.) Feedback resistant MetA (*metA* gene product) mutants in *E. coli* leading to deregulation of methionine biosynthesis were isolated previously by selecting for growth on the toxic methionine analogue α-methyl methionine. (See Usuda and Kurahashi (2005) Appl

Environ Microbiol 71:3228-3234; and International Patent Application Publication No. WO2005/111202.)

The *metK* gene encodes for the enzyme S-adenosylmethionine synthase, which converts methionine to S-adenosylmethionine. (See Markham *et al.*, (1980) J Biol Chem 255:9082-9092.) Partial loss-of-function MetK mutants resulting in low levels of SAM and hence de-repression of methionine biosynthetic enzymes (SAM is a co-repressor for MetJ) were previously isolated by selecting for growth on toxic methionine analogues, norleucine, and ethionine. (See Chattopadhyay *et al.*, (1991) Gen Microbiol 137:685-691; Usuda and Kurahashi (2005) Appl Environ Microbiol 71:3228-3234; and International Patent Application Publication No. WO2005/111202.)

In the present invention, specific nucleic acid residues in the wild-type *metA* gene were mutated, resulting in the following amino acid substitutions in MetA (see Figure 7A and SEQ ID NO:29 for wild-type MetA amino acid sequence): arginine to cysteine substitution at amino acid position 27 (R27C); glutamine to glutamic acid substitution at amino acid position 64 (Q64E); tyrosine to cysteine substitution at amino acid position 294 (Y294C); isoleucine to serine substitution at amino acid position 296 (I296S); and proline to leucine substitution at amino acid position 298 (P298L). *E. coli* host cells comprising one or more of these MetA amino acid substitutions produced methionine to a degree or extent sufficient to result in prevention of norleucine misincorporation in expressed heterologous proteins.

In some embodiments, the present invention provides various mutant *metA* alleles encoding the amino acid substitutions in MetA of R27C, Q64E, Y294C, I296S, and P298L (compared to the wild-type MetA amino acid sequence; Figure 7 and SEQ ID NO:29). Such mutant *metA* alleles resulted in feedback resistant MetA enzyme. The mutant *metA* alleles were introduced into *E. coli* host cells (60E4) using an allele exchange method (see Materials and Methods below) to obtain mutant *E. coli* host cell strain 66H6 (60E4 *metA*(R27C)), 66H8 (60E4 *metA*(Y294C)), 67B8 (60E4 *metA*(Q64E)), and 67B9 (60E4 *metA*(I296S P298L)). The resulting mutant *E. coli* host cells obtained were evaluated for norleucine misincorporation during recombinant protein production performed without a continuous methionine feed. (See Example 4 below.)

All references to amino acid positions in MetA are made based on the homoserine succinyltransferase encoded by the *metA* gene of *E. coli* shown in Figures 7A and 7B, corresponding to SEQ ID NO:29 and SEQ ID NO:31. Reference to amino acid positions are made with the first amino acid methionine counting as amino acid position number 1. The relative positions of corresponding regions in homoserine succinyltransferase enzymes from other organisms can be identified by a person skilled in the art by, for example, simple sequence alignment.

In the present invention, a nucleic acid in the wild-type *metK* gene was mutated, resulting in the amino acid substitution in MetK of valine to glutamic acid at amino acid position 185 (V185E)). (See Figure 8A and SEQ ID NO:30 for wild-type MetK amino acid sequence.) Additionally, a specific nucleic acid was deleted at cytosine base position 1132 in the *metK* gene (c1132del). *E. coli* host cells comprising one or more of these mutant *metK* alleles produced methionine to a degree or extent sufficient to result in prevention of norleucine misincorporation in expressed heterologous proteins.

In some embodiments, the present invention also provides various mutant *metK* alleles encoding the amino acid substitution V185E or a deletion of the cytosine base at position 1132 in the *metK* allele (c1132del). Such mutant *metK* alleles result in partial loss-of-function MetK enzymes. The mutant *metK* alleles were introduced into various *E. coli* host cells (66H8; 60E4 *metA*(Y294C), see above) using an allele exchange method (see Materials and Methods below) to obtain the *E. coli* host cell strains 67C2 (66H8 *metK*(V185E)) and 67C3 (66H8 *metK*(c1132del)), respectively. The resulting mutant *E. coli* host cells obtained were evaluated for norleucine misincorporation during recombinant protein production performed without a continuous methionine feed. (See Example 4 below.)

All references to amino acid positions in MetK are made based on the S-adenosylmethionine synthase encoded by the *metK* gene of *E. coli* shown in Figures 8A and 8B, corresponding to SEQ ID NO:30 and SEQ ID NO:32. Reference to amino acid positions are made with the first amino acid methionine counting as amino acid position number 1. The relative positions

of corresponding regions in S-adenosylmethionine synthase enzymes from other organisms can be identified by a person skilled in the art by, for example, simple sequence alignment.

Nucleic Acid Molecules for *metA* and *metK*

By way of example, the present invention used isolated nucleic acid molecules comprising nucleic acid sequences of *metA* and *metK* that differ from nucleic acid sequences of wild-type *metA* and *metK*. The nucleic acid sequences of *metA* and *metK* provided by the present invention encode various amino acid substitutions to that encoded by wild-type *metA* (arginine at amino acid position 27 replaced with cysteine (R27C); glutamine at amino acid position 64 replaced with glutamic acid (Q64E); tyrosine at amino acid position 294 replaced with cysteine (Y294C); isoleucine at amino acid position 296 replaced with serine (I296S); proline at amino acid position 298 replaced with leucine (P298L); and isoleucine at amino acid position 296 replaced with serine (I296S) and proline at amino acid position 298 replaced with leucine (P298L)); and to that encoded by wild-type *metK* (valine at amino acid position 185 replaced with glutamic acid (V185E) and nucleic acid sequences comprising a deletion of the cytosine base at position 1132 (cdel1132del)). The use of any nucleic acid sequence encoding a *metA* allele or a *metK* allele which result in these amino acid substitutions is specifically contemplated herein for use in the methods of the present invention.

The present invention also provides isolated *metA* nucleic acid molecules encoding various altered MetA enzymes (*i.e.*, encoding various mutant homoserine succinyltransferase enzymes). In some embodiments, the present invention provides an isolated nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25 and SEQ ID NO:26.

The present invention also provides isolated *metK* nucleic acid molecules encoding various altered MetK enzymes (*i.e.*, encoding various mutant S-adenosylmethionine enzymes). In some embodiments, the present invention provides an islolated nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

The present invention also provides, by way of example, various combinations of mutant *metA* alleles and corresponding isolated nucleic acid molecules comprising nucleic acid sequences encoding the following amino acid substitutions in MetA: arginine at amino acid position 27 substituted with cysteine (R27C); glutamine at amino acid position 64 substituted with glutamic acid (Q64E); tyrosine at amino acid position 294 substituted with cysteine (Y294C); isoleucine at amino acid position 296 substituted with serine (I296S); proline at amino acid position 298 replaced with leucine (P298L); and isoleucine at amino acid position 296 substituted with serine (I296S) and proline at amino acid position 298 substituted with leucine (P298L). In some aspects, the mutant *metA* alleles provided by the present invention result in feedback-resistant (*i.e.*, feedback-insensitive) MetA enzymes. Amino acid positions are in reference to wild-type MetA amino acid sequence as shown in Figure 7A and SEQ ID NO:29.

Also provided by the present invention, by way of example, are various combinations of mutant *metK* alleles and corresponding isolated nucleic acid molecules comprising nucleic acid sequence encoding the following amino acid substitution in MetK: valine at amino acid postion 185 substituted with glutamic acid (V185E). The present invention also provides nucleic acid sequences comprising a deletion of the cytosine base at position 1132 (c1132del) of the *metK* allele. In some aspects, the mutant *metK* alleles provided by the present invention result in partial loss-of-function MetK enzymes. Amino acid positions are in reference to wild-type MetK amino acid sequences as shown in Figure 8A and SEQ ID NO:30.

Microorganisms for Use in the Present Methods

As described herein and by way of example, *E. coli* host cells were engineered for the bacteria to produce methionine to a degree or extent sufficient for the prevention or reduction of norleucine misincorporation during recombinant protein production, including recombinant protein production performed at high host cell densities. Accordingly, in some embodiments provided herein, the present invention provides mutant microorganism strains (*i.e.*, mutant microorganism host cells) which produce methionine to a degree or extent sufficient to reduce or prevent norleucine misincorporation into proteins or polypeptides (*e.g.*, to a degree or extent sufficient to reduce or prevent norleucine misincorporation into recombinant proteins

or recombinant polypeptides, or to a degree or extent sufficient to reduce or prevent norleucine misincorporation into heterologous proteins or heterologous polypeptides).

Starting *E. coli* host cells suitable for use in the methods provided herein include, for example, (but are not limited to) *E. coli* W3110, *E. coli* 294, *E. coli* X1776, etc. These examples of *E. coli* host cells are illustrative rather than limiting. *E. coli* strain W3110 is a common host strain for recombinant DNA product fermentations. Mutant *E. coli* host cells of any of the above-mentioned *E. coli* host cell strains may also be employed as the starting host cells that are then further modified to contain the mutated *metA* and/or *metK* alleles described herein.

The present invention shows that use of *E. coli* host cells comprising various mutant alleles and combinations of mutant alleles for *metA* and *metK* in recombinant protein production was effective at preventing norleucine misincorporation into expressed recombinant proteins. (See Example 4 below.)

The present invention provides a microorganism, wherein the microorganism produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into proteins or polypeptides. In some aspects, the present invention provides a microorganism, wherein the microorganism is a feedback-insensitive homoserine succinyltransferase microorganism. In some embodiments, the present invention provides a microorganism comprising a mutant *metA* allele. In other embodiments, the present invention provides a microorganism comprising a mutant *metA* allele, wherein the mutant *metA* allele encodes a R27C amino acid substitution in MetA, a Q64E amino acid substitution in MetA, a Y294C amino acid substitution in MetA, an I296S amino acid substitution in MetA, or a P298L amino acid substitution in MetA. In some embodiments, the present invention provides a microorganism comprising a mutant *metA* allele encoding more than one amino acid substitution described above, including, for example, a mutant *metA* allele encoding a I296S amino acid substitution and a P298L amino acid substitution in MetA. In various aspects, the microorganism comprising any one or more of the nucleic acid sequences provided by the present invention is bacteria; in other aspects, the microorganism is *E. coli*. The present invention specifically provides for the use of microorganisms described herein

for production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced or prevented.

As described above, the present invention provides microorganisms comprising one or more mutant *metA* alleles. In some embodiments, the mutant *metA* alleles encoding a R27C amino acid substitution in MetA, encoding a Q64E amino acid substitution in MetA, encoding a Y294C amino acid substitution In MetA, or encoding a I296S amino acid substitution and a P298L amino acid substitution in MetA, are encoded by a nucleic acid sequence comprising SEQ ID NO:23 (R27C), SEQ ID NO:26 (Q64E), SEQ ID NO:24 (Y294C), or SEQ ID NO:25 (I296S and P298L), respectively. In other embodiments, the microorganisms provided by the present invention comprise mutant *metA* alleles encoded by a nucleic acid sequence comprising SEQ ID NO:23 (R27C), SEQ ID NO:26 (Q64E), SEQ ID NO:24 (Y294C), or SEQ ID NO:25 (I296S and P298L). In various aspects, a microorganism comprising any one or more of the nucleic acid sequences provided by the present invention is bacteria; in other aspects, the microorganism is *E. coli*. The present invention specifically provides for the use of microorganisms described herein for the production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein the misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced or prevented.

As stated above, the present invention provides methods for preventing or reducing norleucine incorporation into proteins and polypeptides expressed by a microorganism, wherein the microorganism is a microorganism which produces methionine to a degree or extent sufficient to prevent or reduce norleucine misincorporation into proteins or polypeptides. In some aspects, the present invention provides a microorganism, wherein the microorganism is a microorganism de-repressed for methionine production. In some embodiments, the present invention provides a microorganism comprising a mutant *metK* allele. In other embodiments, the present invention provides a microorganism comprising a mutant *metK* allele, wherein the mutant *metK* allele encodes a V185E amino acid substitution in MetK. In some embodiments, the present invention provides a microorganism comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a deletion of the nucleic acid

cytosine at nucleic acid residue 1132 of the *metK* allele. In various aspects, a microorganism comprising any one or more of the nucleic acid sequences provided by the present invention is bacteria; in other aspects, the microorganism is *E. coli*. The present invention specifically provides for the use of the microorganisms described herein for the production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein the misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced or prevented.

As described above, the present invention provides microorganisms comprising one or more mutant *metK* alleles. In some embodiments, the mutant *metK* alleles encoding a V185E amino acid substitution in MetK or comprising a deletion of the nucleic acid cytosine at nucleic acid residue 1132 of the *metK* allele, are encoded by a nucleic acid sequence comprising SEQ ID NO:27 (V185E) or a nucleic acid sequence comprising SEQ ID NO:28 (c1132del), respectively. In other embodiments, the microorganisms provided by the present invention comprise mutant *metK* alleles encoded by a nucleic acid sequence comprising SEQ ID NO:27 (V185E) or SEQ ID NO:28 (c1132del). In various aspects, the microorganism comprising any one or more of the nucleic acid sequences provided by the present invention is bacteria; in other aspects, the microorganism is *E. coli*. The present invention specifically provides for the use of any microorganisms described herein for the production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced or prevented.

The use of any nucleic acid sequence encoding *metA* or *metK* alleles which result in the amino acid substitutions described herein are specifically contemplated herein for use in the methods of the present invention.

In other embodiments, the present invention provides a microorganism comprising a mutant *metA* allele and a mutant *metK* allele. In some embodiments, a microorganism provided by the present invention is a microorganism comprising a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele encodes a Y294C amino acid substitution in MetA and the mutant *metK* allele encodes a V185E amino acid substitution in MetK. In some

embodiments provided by the present invention, the microorganism is a microorganism comprising a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele encodes a Y294C amino acid substitution in MetA and the mutant *metK* allele comprises a deletion of the nucleic acid cytosine at nucleic acid residue 1132 of the *metK* allele. In various aspects, a microorganism comprising any one or more of the nucleic acid sequences provided by the present invention is bacteria; in other aspects, the microorganism is *E. coli*. The present invention specifically provides for the use of any microorganisms described herein for the production of heterologous (*e.g.*, recombinant) polypeptides and heterologous (*e.g.*, recombinant) proteins, wherein misincorporation of norleucine into the heterologous polypeptides and heterologous proteins is reduced or prevented.

Production of Microorganism Strains

The present invention provides methods for producing a microorganism (*e.g., E. coli* host cells), wherein the microorganism produces methionine to a degree or extent sufficient to reduce or prevent norleucine misincorporation into polypeptides and proteins. By way of example, *E. coli* host cells comprising mutant *metA* alleles and/or mutant *metK* alleles were generated using allele exchange methods as known in the art and as previously described. (See Metcalf *et al.*, (1994) Gene 138:1-7; and Bass *et al.*, (1996) J Bacteriol 178: 1154-61; see Materials and Methods section of the instant specification.) The present invention is not limited to the means by which *E. coli* host cells comprising mutant *metA* alleles and mutant *metK* alleles are produced. Various methods for introducing mutant alleles or otherwise producing microorganism strains (*e.g.*, bacteria, *E. coli*) comprising mutant alleles are well-known to one skilled in the art.

Prevention or Reduction of Norleucine Misincorporation

Methods and compositions of the present invention can be applied to the production of heterologous or recombinant proteins or polypeptides, and can be used with both large and small scale protein or polypeptide production. Methods and compositions of the present invention are particularly useful for high density fermentation of microorganisms, such as, for example, *E. coli* host cells, for the production of recombinant proteins and polypeptides. The methods and compositions provided by the present invention are useful for recombinant production of proteins and polypeptides, in particular for recombinant production of proteins

and polypeptides in which norleucine misincorporation is undesirable, such as, for example, in recombinant proteins and polypeptides for use in various research and therapeutic applications.

In some embodiments, the present invention provides methods for preventing or reducing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism is a feedback-resistant or feedback-insensitive homoserine succinyltransferase microorganism. In other embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism is a microorganism de-repressed for methionine production. In some embodiments, the feedback-resistant or feedback-insensitive homoserine succinyltransferase microorganism is a microorganism which comprises a mutant *metA* allele. In some embodiments, the microorganism de-repressed for methionine production is a microorganism which comprises a mutant *metK* allele. In other embodiments, the microorganism for use in preventing or reducing norleucine misincorporation comprises a mutant *metA* allele and a mutant *metK* allele.

In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:26, and SEQ ID NO:26. In other embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding MetA, wherein the nucleic acid sequence encodes an amino acid substitution in MetA selected from the group consisting of R27C, Q64E, Y294C, I296S, and P298L. In other embodiments, the nucleic acid sequence encodes amino acid substitutions in MetA consisting of both I296S and P298L. Amino acid positions are in reference to wild-type MetA amino acid sequence as shown in Figure 7A and SEQ ID NO:29.

In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28. In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence encoding MetK, wherein the nucleic acid sequence encodes the amino acid substitution V185E in MetK. In other embodiments, the nucleic acid sequence comprises a deletion of the cytosine base at nucleic acid residue position 1132 in *metK* allele. Amino acid positions are in reference to wild-type MetK amino acid sequence as shown in Figure 8A and SEQ ID NO:30.

In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, and further wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:27. In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises the nucleic acid sequence of SEQ ID NO:24, and the mutant *metK* allele comprises the nucleic acid sequence of SEQ ID NO:28.

In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding the amino acid substitution Y294C in MetA, and the mutant *metK* allele

comprises a nucleic acid sequence encoding the amino acid substitution V185E in MetK. In some embodiments, the present invention provides methods for reducing or preventing norleucine misincorporation in a protein or polypeptide, the method comprising expressing the protein or polypeptide in a microorganism, wherein the microorganism comprises a mutant *metA* allele and a *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence encoding the amino acid substitution Y294C in MetA, and the mutant metK allele comprises a nucleic acid sequence comprising a deletion of the cytosine base at nucleic acid residue position 1132 in *metK* allele. Amino acid positions are in reference to wild-type MetA amino acid sequence as shown in Figure 7A and SEQ ID NO:29 and in reference to wild-type MetK amino acid sequence as shown in Figure 8A and SEQ ID NO:30.

In some aspects of the methods for reducing or preventing norleucine misincorporation into a protein or polypeptide by a microorganism provided herein, the microorganism is a bacteria, in particular an *E. coli*. In other aspects, the protein or polypeptide is a heterologous protein or a heterologous polypeptide, or a recombinant protein or a recombinant polypeptide. For example, the microorganism can comprise a nucleic acid encoding a protein or polypeptide heterologous to the microorganism; *e.g.*, the microorganism is transformed with a nucleic acid encoding a protein or polypeptide heterologous to the microorganism, which can be, for example, DNA (e.g., cDNA or genomic DNA), as by use of a recombinant expression vector. In other aspects, the method further comprises culturing the microorganism under conditions suitable for expression of the protein or polypeptide. In some embodiments, the microorganism is grown in a culture medium, wherein the culture medium contains a low concentration of methionine. The protein or polypeptide can then be recovered, purified, etc; the recovery may be from, for example, the periplasm or culture medium of the microorganism. In some aspects, the culturing takes place in a fermentor, such as, for example, culturing under conditions of high cell-density fermentation.

A heterologous nucleic acid encoding a heterologous protein or polypeptide is suitably inserted into a replicable vector for expression in the microorganism under the control of a suitable promoter. Many vectors are available for this purpose, and selection of the appropriate vector will depend on, for example, the size of the nucleic acid to be inserted into

the vector or the particular microorganism host cell to be transformed with the vector. Suitable vectors are well-known to one of ordinary skill in the art.

The methods and compositions provided by the present invention are particularly useful for production of recombinant proteins and polypeptides in which norleucine misincorporation is undesirable, such as, for example, in recombinant proteins and polypeptides for use in various therapeutic, medical, research, and diagnostic applications. For example, the methods and compositions of the present invention are applicable for recombinant production of therapeutic antibodies, such as, for example, polyclonal and monoclonal antibodies for medical and pharmaceutical use. Examples of polyclonal and monoclonal antibodies for medical and pharmaceutical use include, but are not limited to, anti-VEGF antibodies, anti-factor D antibodies, anti-hepatocyte growth factor receptor antibodies (*e.g.*, anti-MET antibodies), etc.

The methods and compositions of the present invention are also useful for the production of antibody fragments. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (*e.g.*, scFv); and multispecific antibodies formed from antibody fragments. Production of recombinant antibodies as provided by the present methods, compositions, and microorganisms, can be performed by expression of a nucleic acid encoding an antibody heavy chain polypeptide and expression of a nucleic acid encoding an antibody light chain polypeptide within a microorganism as described above (*e.g.*, bacterial host cell, *E. coli*). In some aspects, the antibody heavy chain and the antibody light chain are full-length heavy chain and light chain antibody polypeptides. In other aspects, the antibody heavy chain is an antibody Fab fragment heavy chain, and the antibody light chain is an antibody Fab fragment light chain.

The methods and compositions of the present invention are also useful for the production of multi-specific antibodies, *e.g.*, bispecific antibodies. Multi-specific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary multi-specific antibodies may bind to two different epitopes of the protein, or may bind two different epitopes of two different proteins. Bispecific antibodies can be prepared as full-length

antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antbodies). Techniques for making multi-specific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983); International Application Publication No. WO 93/08829; Traunecker et al., *EMBO J.* 10: 3655 (1991)); and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168).

Additionally, the methods and compositions of the present invention are useful for the production of other biomolecules for therapeutic and research applications, such as, for example, human growth hormone (somatropin), insulin, etc.

EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains used in the Examples described herein are derivatives of the *E. coli* strain W3110. (See Bachmann (1972) Bacteriol Rev 36:525-557.) Antibiotic selection was maintained for all markers at the following concentrations: carbenicillin (plasmid or chromosomal), 50 μg/ml; kanamycin (chromosomal), 30 μg/ml; tetracycline (plasmid or chromosomal), 10 μg/ml.

Strain and plasmid construction

Oligonucleotides used in the construction of plasmids and bacterial strains (i.e., *E. coli*) are listed in Table 1 below. Standard techniques were used for cloning, DNA analysis, PCR amplification, transformation, electroporation, and P1 transduction. Chromosomal alleles were moved by P1 transduction. The *metJ*::Kan^R allele was derived from the bacterial strain JW3909-1, which was obtained from The Coli Genetic Stock Center (CGSC, Yale University). All allele replacements were confirmed by PCR analysis.

Table 1

Primer name	Sequence (5'-3')[a]	
SacI-*metA*flank-F	CACACGAGCTCCTCATTTTGCTCATTAACGTTGG	SEQ ID NO: 1
SalI-*metA*flank-R	CACACGTCGACGCGAATGGAAGCTG	SEQ ID NO: 2
SacI-*metK*flank-F	CACACGAGCTCGTATGCAAAGCAGAGATGC	SEQ ID NO: 3
SalI-*metK*flank-R	CACACGTCGACCGTCATTGCCTTGTTTG	SEQ ID NO: 4
*metA*flankmid-F	GTTCTGATCCTTAACCTGATGCCGAAGAAG	SEQ ID NO: 5
*metA*flankmid-R	CCAGCGTTTGCGCATCATATTCGG	SEQ ID NO: 6
*metK*flankmid-F	GGCAAAACACCTTTTTACGTCCGAGTCC	SEQ ID NO: 7
*metK*flankmid-R	GAACTCACGTACCAGCAGGGTCAGTTG	SEQ ID NO: 8
pS1080-P	CCAGTCACGACGTTGTAAAACGACGG	SEQ ID NO: 9
pS1080-T	AGTGAACGGCAGGTATATGTGATGG	SEQ ID NO: 10
QC-*metA*R27C-F	GTGATGACAACTTCTtGTGCGTCTGGTCAGG	SEQ ID NO: 11
QC-*metA*R27C-R	CCTGACCAGACGCACaAGAAGTTGTCATCAC	SEQ ID NO: 12
QC-*metA*Q64E-F	CAAACTCACCTTTGgAGGTCGATATTCAGC	SEQ ID NO: 13
QC-*metA*Q64E-R	GCTGAATATCGACCTcCAAAGGTGAGTTTG	SEQ ID NO: 14
QC-*metA*Y294C-F	GCTCAACTATTACGTCTgCCAGATCACGCCATACG	SEQ ID NO: 15
QC-*metA*Y294C-R	CGTATGGCGTGATCTGGcAGACGTAATAGTTGAGC	SEQ ID NO: 16
QC-metAI296SP298L-F	CGTCTACCAGAgCACGCtATACGATCTACG	SEQ ID NO: 17
QC-*metA*I296SP298L-R	CGTAGATCGTATaGCGTGcTCTGGTAGACG	SEQ ID NO: 18
QC-*metK*V185E-F	ATCGATGCTGTCGaGCTTTCCACTCAG	SEQ ID NO: 19
QC-*metK*V185E-R	CTGAGTGGAAAGCtCGACAGCATCGAT	SEQ ID NO: 20
QC-*metK*c1132del-F	GCGCAGCTGCTGGCGATGCTGCCG	SEQ ID NO: 21
QC-*metK*c1132del-R	CGGCAGCATCGCCAGCAGCTGCGC	SEQ ID NO: 22

[a] Underlined nucleic acid residues introduce amino acid mutations. Lowercase nucleic acid residues indicate residues different from the wild-type nucleic acid sequence.

The *metA* gene was PCR amplified from bacterial strain W3110 (Bachmann (1972) Bacteriol Rev 36:525-557) using primers SacI-*metA*flank-F and SalI-*metA*flank-R, digested with SacI and SalI, and ligated into SacI and SalI digested plasmid pS1080 to obtain plasmid pS1080-*metA*flank. Plasmids pS1080-*metA*flank(R27C), pS1080-*metA*flank(Q64E), pS1080-*metA*flank(Y294C), and pS1080-*metA*flank(I296SP298L) were constructed by mutagenizing plasmid pS1080-metAflank using a QuikChange kit (Stratagene) and the following sets of primers: (QC-*metA*R27C-F;QC-*metA*R27C-R), (QC-*metA*Q64E-F;QC-*metA*Q64E-R), (QC-

*metA*Y294C-F;QC-*metA*Y294C-R) and (QC-*metA*I296SP298L-F;QC-*metA*I296SP298L-R), respectively.

The *metK* gene was PCR amplified from bacterial strain W3110 using primers SacI-*metK*flank-F and SalI-*metK*flank-R, digested with SacI and SalI, and ligated into SacI and SalI digested plasmid pS1080 to obtain plasmid pS1080-*metK*flank. Plasmids pS1080-*metK*flank(V185E) and pS1080-*metK*flank(c1132del) were constructed by mutagenizing plasmid pS1080-*metK*flank using a QuikChange kit (Stratagene) and the following set of primers: (QC-*metK*V185E-F;QC-*metK*V185E-R), and (QC-*metK*c1132del-F;QC-*metK*c1132del-R), respectively.

Allele exchange was carried out using the methods previously described. (See Metcalf *et al.*, (1994) Gene 138:1-7; and Bass *et al.*, (1996) J Bacteriol 178: 1154-61.)

As stated above, allele exchange was conducted using the protocol described by Metcalf *et al.* (*supra*) as modified by Bass *et al.* (*supra*). Cointegrates were transferred into 60E4 host cell background or 66H8 host cell background. Following sucrose counter-selection, sucrose-resistant colonies were screened for carbenicillin sensitivity by replica streaking on LB agar and LB agar plates containing carbenicillin. Carbenicillin-sensitive colonies were subsequently isolated and allele exchange was confirmed by PCR amplification of the entire *metA* or *metK* reading frame followed by DNA sequencing. The suicide plasmid vector pS1080 contains the conditional R6Kγ origin and carbenicillin resistance selectable marker, as well as a counter-selectable *sacB* gene, which confers sucrose sensitivity.

Bacteria strains and plasmids used in the experiments described herein are listed in Table 2 below.

Table 2

Strain or plasmid	Genotype or description	Ref. or source
W3110	F^- lambda$^-$ IN(*rrnD-rrnE*)1 *rph-1*	Laboratory collection
JW3909-1	(Δ*araD-araB*)567 Δ*lacZ*4787(::*rrnB-3*) λ^- *rph-1* Δ(*rhaD-rhaB*)568, Δ*metJ*725::kan^R *hsdR514*	CGSC

60E4	W3110 Δ*fhuA* (Δ*tonA*) Δ*ptr* Δ*ompT* Δ*degP* Δ*phoA ilvG+* Δ*fuc* Δ*rha*	Laboratory collection
66G6	60E4 *ΔmetJ725::kan*^{R}	This study
66H6	60E4 *metA(R27C)*	This study
66H8	60E4 *metA(Y294C)*	This study
67B8	60E4 *metA(I296SP298L)*	This study
67B9	60E4 *metA(Q64E)*	This study
67C2	60E4 *metA(Y294C) metK(V185E)*	This study
67C3	60E4 *metA(Y294C) metK(c1132del)*	This study
66F8	W3110 Δ*fhuA (*Δ*tonA)* Δ*phoA ilvG2096* (IlvG^{+}; Val^{r}) Δ*manA* Δ*prc spr43H1* Δ*degP lacI*^{q} Δ*ompT* Δ*menE*	Laboratory collection
67C5	66F8 *metA(Y294C)*	This study
64B4	W3110 Δ*fhuA (*Δ*tonA)* Δ*phoA ilvG2096* (IlvG^{+}; Val^{r}) Δ*manA* Δ*prc spr43H1* Δ*degP lacI*^{q} Δ*ompT*	Laboratory collection
67C4	64B4 *metA(Y294C)*	This study
pS1080	Counter-selectable allele-exchange suicide vector, R6Kγ origin, SacB, Carb^{r}	Laboratory collection
pS1080-*metA*	MetA in pS1080	This study
pS1080-*metK*	MetK in pS1080	This study
pS1080-*metA(R27C)*	MetA with R27C mutation cloned in pS1080	This study
pS1080-*metA(Q64E)*	MetA with Q64E mutation cloned in pS1080	This study
pS1080-*metA(Y294C)*	MetA with Y294C mutation cloned in pS1080	This study
pS1080-*metA(I296SP298L)*	MetA with I296S and P298L mutations cloned in pS1080	This study
pS1080-*metK(V185E)*	MetK with V185E mutation cloned in pS1080	This study
pS1080-*metK(c1132del)*	MetK with cytosine deletion at position 1132 cloned in pS1080	This study

Fermentation

The *E. coli* host strain 60E4 was transformed with a pBR322-based expression plasmid containing polynucleic acid encoding a light chain and a heavy chain of an anti-VEGF antibody antigen binding (Fab) fragment (SEQ ID NO:33 and SEQ ID NO:34, respectively). (See anti-VEGF antibody Y0317 in International Application Publication No. WO1998/45331; International Application Publication No. WO2002/40697 (Example 2, describing fermentation of anti-VEGF antibody Y0317); and Chen et al., (1999) J Mol Biol 293:865-881, anti-VEGF antibody Y0317, each of which is incorporated herein in its entirety

by reference.)

The *E. coli* host strain 66F8 was transformed with an expression plasmid containing polynucleic acid encoding a light chain and a heavy chain of an anti-Factor D antibody antigen binding (Fab) fragment, corresponding to the amino acid sequence of SEQ ID NO:48 and SEQ ID NO:49, respectively. (See anti-Factor D antibody number 238-1 in International Application Publication No. WO2009/134711 and anti-Factor D antibody number 111 in International Application Publication No. WO2008/055206, each of which is incorporated herein in its entirety by reference.)

The *E. coli* host strain 64B4 was transformed with an expression plasmid containing polynucleic acid encoding a light chain, a heavy chain, and a heavy chain fragment of an anti-MET antibody corresponding to the amino acid sequence of SEQ ID NO:50, SEQ ID NO:51, and SEQ ID NO:52, respectively).

Expression of the recombinant heavy chain and light chain Fab fragment polypeptides was controlled by the *phoA* promoter with induction occurring upon the depletion of inorganic phosphate in the medium. (See Laird *et al.*, (2005) Protein Expr Purif 39:237-246.) The heavy chain and light chain Fab fragment polypeptides were directed for exportation to the *E. coli* periplasm by a STII-signal sequence, where the product was assembled. High cell density fermentations at the 10 L working volume (WV) were carried out as described previously. (See Simmons *et al.*, (2002) J Immunol Methods 263:133-147.) At a cell density of approximately 200 OD_{550}, a continuous 3% methionine or water feed was initiated and fed through the remainder of the fermentation process.

Three different fermentation processes were examined using host cell 60E4 (fermentation process AF1), host cell 66F8 (fermentation process AF2), and host cell 64B4 (fermentation process AF3). (See Example 5 and Table 4 below.)

Purification

After fermentations were completed, whole cell broth was cooled to <15°C in the fermenter and the cooled broth was processed for protein purification. One volume of the cooled broth

was mixed with 0.06 volumes of $MgSO_4$ (60 mM final concentration) and titrated to pH 3.8 with citric acid (1 M). Cells were then disrupted using a microfluidizer at approximately 12,000 psi (Microfluidics, Redwood Shores, CA) and the disrupted cells were incubated at 35°C for 3 hours with continuous shaking. The homogenate was diluted 3-fold with cold purified water and the diluted homogenate was centrifuged at 6,000×g using a fixed angle rotor at 4°C for 20 minutes. The supernatant was filtered using 0.22 μm filters and titrated to pH 7.5 with 1.5 M Tris base.

The recombinant Fab protein was purified using Protein G affinity chromatography as follows. Poly-prep chromatography columns (Bio Rad) were packed with Protein G Sepharose 4 Fast Flow resin (GE Healthcare) and equilibrated with at least 5 column volumes of PBS, pH 7.2. Filtered supernatant was loaded into the Protein G packed column, washed twice with PBS, and eluted with 50 mM citric acid. The final Fab protein pool was titrated to pH 7 with 1.5 M Tris base and analyzed for norleucine content as described below. This corresponds to purification for fermentation process AF1.

Three different recombinant protein product purification processes were used, each specific to fermentation process AF1 (for host cell 60E4), AF2 (for host cell 66F8), or AF3 (for host cell 64B4). (See Example 7 and Table 6 below.)

Amino acid analysis

To determine intracellular methionine levels, whole cell broth samples containing 87.6×10^9 cells were pelleted at 17,000×g for 5 minutes at 4°C, washed once in PBS, and then resuspended in extraction buffer (10 mM Tris, 5 mM EDTA, 5 mM iodoacetamide (IAM), 0.2 mg/ml lysozyme, pH 6.8). Cells were then lysed by two cycles of sonication, and then centrifuged for 20 min at 13,500 rpm to remove cell debris. The supernatants were transferred to 0.2 μm microcentrifuge tube filters (Bio Rad) and centrifuged at 17,000×g for 5 minutes at 4°C. The filtrates were diluted and the amino acids were analyzed as previously described (Feeney et al., (2013) Biotechnology and Bioengineering, 110:1087-1097). For determining extracellular methionine levels, supernatant samples prepared from whole cell broth collected during fermentation after centrifugation for 3 min at 14,000×rpm were diluted

and the amino acids analyzed as described below. (See Feeney et al., (2013) Biotechnology and Bioengineering, 110:1087-1097.)

Amino acid concentrations were analyzed using a reversed-phase HPLC method. Samples containing amino acids were treated with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to produce highly fluorescent derivatives. (See Cohen and Michaud (1993) Anal Biochem 211:279-287.) HPLC assays used detected the following amino acids with a detection limit of 0.01 mM: histidine, asparagine, serine, glutamine, arginine, glycine, aspartate, glutamate, threonine, alanine, proline, ornithine, cysteine, lysine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and tryptophan.

Phosphate levels

Phosphate levels were measured using COBAS Integra 400 (Roche Diagnostics) according to methods previously published. (See Taussky and Shorr (1953) J Biol Chem 202:675-685.)

Titer measurements

Whole cell broth samples were diluted 6-fold with extraction buffer (10 mM Tris, 5 mM EDTA, 5 mM IAM, 0.2 mg/ml lysozyme, pH 6.8) and incubated for 10 minutes on ice. After two rounds of sonication, the samples were centrifuged at 17,000×g for 20 minutes at 4°C. Product titer was determined from supernatants using HPLC.

Integrated OD_{550} measurements

Integrated OD_{550} was determined by using trapezoidal integration using the following formula:

$$iOD_{550} = \sum_{i=j}^{i=k} (t_i - t_{i-1}) \frac{(OD_{550,i} + OD_{550,i-1})}{2}$$

where,

j = index of the first measurement performed at or after 24 hours of culture time; k = total number of OD_{550} measurements performed; t_i = elapsed culture time in hours at measurement i; $OD_{550,i}$ = OD_{550} at measurement i.

Norleucine analysis

For analysis of norleucine content, purified recombinant protein samples were subjected to trypsin digestion based on a method previously described. (See Yu *et al.*, (2009) Anal Chem 81:9282-9290.) Peptide map analysis was performed using a reversed-phase HPLC and online liquid chromatography tandem mass spectrometry (LC/MS) as previously described. (See Yu *et al.*, (2009) Anal Chem 81:9282-9290; and Yu *et al.*, (2011) Anal Chem 83:5912-5919.) High resolution mass determination was performed with an LTQ-Orbitrap XL instrument (Thermo Scientific, San Jose, US) using a full-MS survey scan with resolution set at 60,000 at m/z 400, followed by ion trap MS2 scans for ions of interest. For determination of the relative level of norleucine within the polypeptides, extracted ion chromatograms were generated for both methionine-containing and norleucine-containing peptides using the most abundant charge state with an extraction window of monoisotopic m/z ± 10 ppm. Relative amount of norleucine-containing species relative to that of methionine-containing species were calculated using the respective integrated peak areas.

Western blots

Whole cell broth samples obtained during the fermentation were diluted 6-fold with extraction buffer (10 mM Tris, 5 mM EDTA, 5 mM IAM, 0.2 mg/ml lysozyme, pH 6.8) and incubated for 10 minutes on ice. After two rounds of sonication, samples were centrifuged at 17,000×g for 25 minutes at 4°C. Samples were loaded on 4-12% Tris-Glycine gels under non-reducing conditions. Protein was transferred to nitrocellulose membranes using an iBlot Blotting System (Invitrogen). Membranes were blocked with 0.5% Gelatin in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% TritonX-100) for 30 minutes, followed by incubation in a 1:300,000 dilution of peroxidase conjugated goat IgG fraction to human IgG Fab (MP Biomedical) in the blocking buffer. After washing 3 times with NET buffer, the blots were visualized on X-ray film using Western Lightning ECL Substrate (PerkinElmer) after a 5 second exposure.

Example 1. Norleucine misincorporation during *E. coli* fermentation

As described above, norleucine misincorporation often occurs during recombinant protein production in *E. coli*. The extent of norleucine misincorporation during recombinant protein production depends on several factors, such as, for example, the nature of the recombinant

protein, the fermentation process used, and the contents of the fermentation medium. (See, *e.g.*, Bogosian *et al.*, (1989) Biol Chem 264:531-539.)

To examine norleucine misincorporation in a recombinant protein expression fermentation process, the following study was performed. The *E. coli* host strain 60E4 was transformed with a plasmid containing nucleic acid sequences encoding a light chain and a heavy chain of an Fab antibody fragment (SEQ ID NO:31 and SEQ ID NO:32, respectively) and used in the following fermentation studies using a water feed or methionine feed according to methods described above. The expressed recombinant proteins were then analyzed for norleucine content using methods described above.

As shown in Table 3 below, approximately 5-10% norleucine misincorporation was observed in each of the recombinant polypeptides expressed in the *E. coli* host cell 60E4 in the absence of a continuous methionine feed (*i.e.*, a water feed). As expected, in the presence of a continuous methionine feed, norleucine was not detected (ND) in either expressed recombinant polypeptide.

Table 3

Strain	**Feed**	**Norleucine in peptide 1 (%)**	**Norleucine in peptide 2 (%)**
60E4	Met	ND	ND
60E4	Water	5.1 ± 0.7	10 ± 1.2
60E4	No feed		
66H6	Water	ND	ND
66H8	Water	ND	ND
66H8	No feed	ND	ND
67B8	Water	ND	ND
67B9	Water	ND	ND
67C2	Water	ND	ND
67C3	Water	ND	ND

These results confirmed that norleucine misincorporation occurred in recombinant protein production in bacteria in the absence of a methionine feed.

Example 2. Construction of methionine biosynthetic pathway mutant *E. coli* host cells

As stated above, continuous feeding of methionine during recombinant protein fermentation is often used to prevent norleucine misincorporation. As shown above in Example 1, continuous methionine feed ensured that sufficient methionine was available for the host cell, thus reducing or preventing norleucine misincorporation during recombinant protein production. To examine the effect of using an *E. coli* host cell containing mutant *metA* and/or *metK* alleles on norleucine misincorporation, instead of using a continuous methionine feed, the following studies were performed.

In the present studies, *metA* alleles containing the mutations R27C, Q64E, Y294C, I296S, and P298L, which result in feedback-resistant MetA, were introduced into 60E4 host cells using an allele exchange method (see Materials and Methods above) to obtain bacterial host cell strains 66H6 (60E4 *metA*(R27C)), 66H8 (60E4 *metA*(Y294C)), 67B8 (60E4 *metA*(Q64E)), and 67B9 (60E4 *metA*(I296S P298L)), respectively (See Tables 2 and 3 above.)

The *metK* alleles containing the mutations V185E and c1132del (deletion of the cytosine base at position 1132 in the *metK* gene), which result in partial loss of function MetK enzymes, were introduced into 66H8 (60E4 *metA*(Y294C)) host cells using an allele exchange method (see Materials and Methods above) to obtain bacterial host cell strains 67C2 (66H8 *metK*(V185E)) and 67C3 (66H8 *metK*(c1132del)), respectively. (See Tables 2 and 3 above.)

These *E. coli* host cells were evaluated for norleucine misincorporation during recombinant protein production in a fermentation process performed without a continuous methionine feed. (See Example 3 below.)

Example 3. Fermentation Results

Small-scale fermentations (10 L) without a continuous methionine feed were executed utilizing the methionine biosynthetic pathway mutant bacterial strains constructed in this study. (See Table 1.) The methionine feed was either replaced with water feed or no feed was used during the fermentation process in these experiments. Three 10 L fermentations were performed using the control host cell strain 60E4 as follows: 1) a continuous methionine feed, 2) a continuous water feed, and 3) no feed.

Fermentation trends for cell growth, as monitored by OD_{550}, are shown in Figure 12A. Irrespective of the nature of the feed (methionine, water, or no feed), the growth of the methionine biosynthetic pathway mutant bacteria host cells 60E4 *metA*(R27C), 60E4 *metA*(Y294C), 60E4 *metA*(Y294C) *metK*(V185E), and 60E4 *metA*(Y294C) *metK*(c1132del) was comparable to that observed in control host cells during the growth phase of the fermentation (5-28 hours). However, double mutant host cells 60E4 *metA*(Y294C) *metK*(V185E), and 60E4 *metA*(Y294C) *metK*(c1132del) had lower iOD_{550} (area under the growth curve from 24 hours until the end of fermentation) compared to that observed in control host cell fermentations. (See Figure 12B.) Fermentations performed with water feed using host cells 60E4 and 60E4 *metA*(Y294C) had slightly higher iOD_{550} compared to that observed in control host cell fermentation performed with a methionine feed and 60E4 *metA*(Y294C) host cell fermentation performed with no feed, respectively. The mutant cells 60E4 Δ*metJ*::kan^R and 60E4 *metA*(I296S P298L) had longer adaptation phases and as a result had lower iOD_{550} compared to that observed in control host cell fermentations. (See Figures 12A and 12B.) The mutant host cell 60E4 *metA*(Q64E) grew poorly in the fermentor, reaching a maximum OD_{550} of 150, which is approximately 30-40% lower compared to the maximum OD_{550} observed in fermentations using other mutant host cells. (See Figure 12A.) After 20 hours, growth of mutant host cell 60E4 *metA*(Q64E) reached saturation and as a result, the fermentation using this mutant host cell had the lowest iOD_{550}. (See Figures 12A and 12B.)

The presence or absence of a methionine feed during the fermentation did not affect growth of the 60E4 host cells. (See Figure 12C.)

Fermentations using the methionine biosynthetic pathway mutants accumulated higher levels of methionine both *in vivo* (*i.e.*, intracellular) and in the extracellular medium compared to that observed in control host cell fermentation. (See Figures 13A, 13B, 14A, and 14B.) At the beginning of the fermentation process, there is excess methionine (>3 mM) in the fermentation medium. As the cells begin to grow, they uptake methionine for protein synthesis, methyl donor role, and other functions. As a result, the extracellular methionine concentration gradually decreases as the cells continue to grow and the extracellular

methionine levels reach less than detectable levels (<10 μM) at approximately hour 16. (See Figures 13A and 14A.)

At hour 16, the intracellular methionine concentrations vary from 0.5-2.5 mM (concentration is based on cell volume) among different hosts. (See Figures 13B and 14B.) At such high intracellular methionine concentrations, wildtype MetA would be strongly inhibited; however, feedback resistant MetA mutants may only be weakly inhibited and thus allowing the mutant host cells to produce methionine via the biosynthetic pathway. (See Usuda and Kurahashi (2005) Appl Environ Microbiol 71:3228-3234.) However, the intracellular methionine levels continued to decrease until about hour 28, at which point cell growth was considerably slowed down. It is possible that during the bacterial growth phase (5-28 hours) of the fermentations, the rate at which the methionine is utilized for protein synthesis and other cellular functions may exceed the rate at which methionine is synthesized *in vivo*. This may explain the gradual decrease in intracellular methionine levels until the end of growth phase.

During the recombinant protein production phase of the fermentation (28 hours until the end of the fermentation), the methionine overproducing host cells continue to synthesize methionine *in vivo* and the intracellular methionine levels continued increasing during this phase of the fermentation process. (See Figures 13B and 14B.) These results suggested that during the recombinant protein production phase of the fermentation, the rate of methionine biosynthesis exceeded the rate at which methionine was utilized for various intracellular functions.

During control host cell fermentation performed with a continuous water feed, both the extracellular and intracellular methionine levels continued to decrease, reaching levels less than the limit of detection of the assay (10 μM) at about 16 hours for extracellular methionine and at about 24 hours for intracellular methionine. For a control host fermentation performed with a continuous methionine feed, the feed ensures that there is excess methionine in the cell after approximately 26 hours, the time point at which the feed is initiated. During the production phase of fermentation, the double mutant host cell 60E4 *metA*(Y294C)

metK(V185E) accumulated more intracellular methionine compared to that observed in control host cell fermentation performed with a continuous methionine feed.

Longer adaptation phase of host cells 60E4 *metA*(I296S P298L) and 60E4 Δ*metJ*::kan^R and poor growth of host cell 60E4 *metA*(Q64E) compared to that observed in control host cells could potentially be due to high levels of homocysteine accumulation, a toxic intermediate in the methionine biosynthetic pathway. (See Roe *et al.*, (2002) Microbiology 148:2215-2222; See Figure 12A.) It was demonstrated previously that homocysteine inhibits the enzyme involved in the first step of isoleucine biosynthetic pathway, threonine deaminase, causing growth inhibition. (See Tuite *et al.*, (2005) J Bacteriol 187:4362-4371.) This was examined by measuring the intracellular isoleucine levels in the mutant host cells. The analysis showed that the intracellular isoleucine levels were comparable to that observed in control host cells during fermentation (data not shown). The possibility of homocysteine having other toxic effects on cell growth cannot be completely ruled out. At this time, however, these differences in growth among the mutants are not fully understood.

Time course for protein product titers and western blot data are shown in Figures 16A, 16B, and 17. The fermentation inoculated from host cell 60E4 *metA*(Q64E) produced less product that that observed in other host cells. Except for a brief period between 45-50 hours, phosphate levels never depleted during the 60E4 *metA*(Q64E) host fermentation (Figure 15); thus, recombinant protein synthesis was low. The extended adaptation phase of the mutant host cells 60E4 *metA*(I296S P298L) and 60E4 Δ*metJ*::kan^R resulted in phosphate depletion after hour 40 which is about 12 hours later than as usually observed; hence, fermentations using these host cells had lower protein product titers compared to that observed in other mutant host cells that depleted phosphate earlier. (See Figures 12A, 15 and 16A.) Fermentations using the host cells 60E4 *metA*(R27C) and 60E4 *metA*(Y294C) produced the highest protein product titers among all the mutant host cells examined.

Fermentations using the *metA metK* double mutant host cells, 60E4 *metA*(Y294C) *metK*(V185E) and 60E4 *metA*(Y294C) *metK*(c1132del), produced somewhat low protein product titers in spite of having comparable growth to control host cells. These double mutant host cells have a mutation in the *metK* gene that results in a partial loss of function MetK. The product of MetK is s-adenosylmethionine (SAM), a methyl donor for many

reactions in bacterial cells. It is not known, however, why decreased SAM levels would affect protein product titers. A continuous feed during the fermentation process could result in dilution of the culture medium that could possibly result in lower cell densities and possibly lower product titers. The growth and titers of host cell 60E4 *metA*(Y294C) fermentation without any feed was comparable to that observed in fermentations using the same host cell performed with a continuous water feed.

Example 4. Norleucine misincorporation

As described above, norleucine misincorporation in proteins due to the levels of methionine in the cell are low enough that norleucine can compete for methionine residues in the charging of methionyl tRNA during protein synthesis. As shown in Example 1 above, control host cell fermentation performed without a methionine feed resulted in high levels of norleucine misincorporation in the recombinant protein (Table 3). Low intracellular methionine levels during the production phase of control host cell fermentation performed without a methionine feed indicated that norleucine residues could be competing for methionine residues in the recombinant protein. However, high levels of extracellular and intracellular methionine were observed during the production phase of the mutant host cell fermentations. (See Figures 13B and 14B.) As a result of elevated intracellular methionine levels, norleucine misincorporation is expected to be minimal or eliminated in using such host cell fermentations.

Trypsin digestion of the recombinant protein yielded 2 methionine containing peptides: peptide 1: LSCAASGYDFTHYG**M**^{34}NWVR (SEQ ID NO:35); and peptide 2: STAYLQ**M**^{83}NSLR (SEQ ID NO:36). Peptide map analysis indicated that the recombinant protein pools purified from the mutant host cell fermentations contained less than detectable levels of norleucine misincorporation, while the control host cell fermentation performed without a methionine feed accumulated high levels of norleucine in both methionine containing peptides. (See Table 3.) These results showed that use of *E. coli* host cell strains of the present invention resulted in the reduction or prevention of norleucine incorporation into heterologous (*e.g.*, recombinant) polypeptides.

Example 5. Additional bacteria host cells

In addition to experiments performed using host cell 60E4 or host cells derived from 60E4, two other bacteria host cells were developed and examined for growth, norleucine misincorporation, and recombinant protein production as follows.

Bacteria host cells 66F8 and 64B4 (as well as bacteria host cell 60E4) are described above in Table 2. As shown in Table 2, there are several differences in the genotype of host cells 60E4 compared to host cells 66F8 and 64B4 (which share a similar genotype).

Three different fermentation processes were examined using host cell 60E4 (fermentation process AF1), host cell 66F8 (fermentation process AF2), and host cell 64B4 (fermentation process AF3). Table 4 below shows the differences in various fermentation parameters (pH, agitation, culture duration, and feed start time) of each of the fermentation processes (AF1, AF2, and AF3) examined.

Table 4

	AF1	AF2	AF3
Control host	60E4	66F8	64B4
pH	7.0	7.0	7.3
Agitation (rpm)	850[a]	650	650
Culture duration (hours)	72	50	72
Feed (met or water) start timing (OD_{550})	200	150	150

[a]After the cells reach an OD_{550} of 200, agitation is reduced to 800 and then stepwise by 100 rpm every 2 hours until 500 rpm is reached.

The *metA*(Y294C*)* allele was introduced into the host cells 66F8 and 64B4 using methods as described above in Example 1 for host cell 60E4. Fermentations performed using the 66F8 *metA*(Y294C*)* and the 64B4 *metA*(Y294C), using fermentation process AF2 and AF3, respectively, showed host cell growth comparable to that observed with their parent host cells. (See Figures 19A and 19B; and Table 5 below.)

Example 6. Comparison of *E. coli* host cell growth rates and recombinant protein product yields

Growth rates and recombinant protein product yields were examined in each of the *E. coli* host strains 60E4 *metA*(Y294C), 66F8 *metA*(Y294C), and 64B4 *metA*(Y294C) using fermentation process AF1, AF2, AF3, respectively. 10L fermentations were performed as described above in Example 3 for host cells of strain 60E4, using fermentation process modifications as outlined above in Table 4 for each fermentation process.

Growth rates and recombinant protein product yields observed for the various host cells of strain 60E4 are discussed in detail above in Example 3.

As shown in Figures 20A, 20B, and 20C, recombinant protein product yields obtained using host strains 60E4 *metA*(Y294C), 66F8 *metA*(Y294C), and 64B4 *metA*(Y294C) was comparable to that observed using host strains 60E4, 66F8, and 64B4. (See also Table 5 below.) The presence or absence of methionine feed did not affect recombinant protein yields obtained from the 60E4 host cell fermentation.

Table 5

Host	Feed	Process	Growth rate, μ (hr^{-1})[a]	Product yield (g/L)[b]
60E4 (control host)	met	AF1	0.325 ± 0.02	1.077 ± 0.047
60E4 (control host)	water	AF1	0.343	1.152
60E4 (control host)	none	AF1	0.358 ± 0.15	1.098
60E4 *metJ*	water	AF1	0.232	0.486
60E4 *metA(R27C)*	water	AF1	0.332	0.792
60E4 *metA(Y294C)*	water	AF1	0.333	0.906
60E4 *metA(Y294C)*	none	AF1	0.377 ± 0.023	0.924 ± 0.017
60E4 *metA(Q64E)*	water	AF1	0.294	0.102
60E4 *metA(I296S P298L)*	water	AF1	0.278	0.57

60E4 *metA(Y294C) metK (V185E)*	water	AF1	0.356	0.696
60E4 *metA(Y294C) metK (V185E)*	water	AF1	0.301	0.618
60E4 *metA(Y294C)*	none[c]	AF1	0.314	0.936
66F8 (control host)	met	AF2	0.459 ± 0.025	4.5 ± 0.5
66F8 *metA(Y294C)*	none	AF2	0.381 ± 0.03	6.7 ± 0.2
64B4 (control host)	met	AF3	0.406 ± 0.037	5.1 ± 0.2
64B4 *metA(Y294C)*	none	AF3	0.361 ± 0.01	5.4 ± 0.1

[a]For the 60E4 *metJ* and 60E4 metA *metA(I296S P298L)* hosts, the time between 6-14 hours and 14-22 hours respectively was used to calculate μ. For all other hosts, 2-10 hours was used to calculate μ. The values of μ shown are the average of n=2 runs.
[b]The values shown are the average of n=2 runs.
[c]Norleucine bolus at a final concentration of 0.15 mM was added during the fermentation when the cells reached an OD_{550} of 200.

Example 7. Comparison of norleucine misincorporation

Three different recombinant protein product purification processes were used, each specific to fermentation process AF1 (for host cell 60E4), AF2 (for host cell 66F8), and AF3 (for host cell 64B4). Table 6 below shows the differences in the various purification processes used for each of the fermentation processes (*i.e.,* AF1, AF2, and AF3) examined.

Table 6

	AF1	AF2	AF3
Flocculant[a]	$MgSO_4$ (50 mM)	$MgSO_4$ (50 mM)	Polyethyleneimine (0.4%)
Homogenate hold	3 hours at 35°C	21 hours at 30°C	12 hours at 30°C
Affinity resin	Protein G	Protein G	MabSelect Sure
Elution buffer	Citric acid (50 mM)	Citric acid (50 mM)	Glycine phosphate (75 mM), pH 3.3

[a]Final concentrations are indicated for the flocculant

Norleucine quantification was performed using LC-MS analysis on tryptic peptides for each of the recombinant protein products as described above.

Trypsin digestion of the recombinant protein produced by the 60E4 host yielded 2 methionine-containing peptides (Table 7). Trypsin digestion of the recombinant protein produced by the 66F8 host yielded 3 methionine-containing peptides (Table 8). Trypsin digestion of the recombinant protein produced by the 64B4 host yielded 6 methionine-containing peptides (Table 9).

Table 7

Tryptic peptides	SEQ ID NO:	60E4 met feed	60E4	60E4 *metA*(Y294C)
STAYLQ**M**NSLR	36	ND	10 ± 1.2	ND
LSCAASGYDFTHYG**M**NWVR	35	ND	5.1 ± 0.7	ND

Table 8

Tryptic peptides	SEQ ID NO:	66F8 met feed	66F8	66F8 *metA*(Y294C)
ASGYTFTNYG**M**NWVR	37	ND	2.1 ± 0.4	ND
QAPGQGLEW**M**GWINTYTGETTYADDFK	38	ND	0.7 ± 0.2	ND
VTITCITSTDIDDD**M**NWYQQKPGK	39	ND	1 ± 0.3	ND

Table 9

Tryptic peptides	SEQ ID NO:	64B4 met feed	64B4	64B4 *metA*(Y294C)
GLEWVG**M**IDPSNSDTR	40	ND	1.3 ± 0.3	ND
NTAYLQ**M**NSLR	41	ND	1.3 ± 0.3	ND
DTL**M**ISR	42	ND	2.4 ± 0.4	ND
EE**M**TK	43	ND	2.2 ± 0.5	ND
WQQGNVFSCSV**M**HEALHNHYTQK	44	ND	1.5 ± 0.4	ND
DIQ**M**TQSPSSLSASVGDR	45	ND	1.3 ± 0.2	ND

Recombinant protein purified from the AF1 fermentation process performed without a methionine feed using the 60E4 host accumulated 5.1% and 10% norleucine at the two methionine residues in the protein (Table 7). No norleucine was detected in the recombinant protein purified from the AF1 fermentations performed without a methionine feed using the hosts 60E4 *metA(Y294C)* (Table 7), 60E4 *metA(R27C)*, 60E4 *metA(Y294C) metK(V185E)*, and 60E4 *metA(Y294C) metK(c1132del)* (data not shown).

When the 60E4 *metA(Y294C)* host fermentation was supplemented with norleucine (0.15 mM final concentration) in the fermentation medium, no norleucine was observed in the recombinant protein, indicating that the bacterial host cells of the present invention make enough methionine in the cell to prevent norleucine misincorporation during recombinant protein synthesis.

About 2.7%, 0.7%, and 1% norleucine misincorporation was observed in the three methionine-containing tryptic peptides obtained from the recombinant protein produced using AF2 fermentation process performed without methionine feed using the 66F8 host. (See Table 8.) Similarly there was about 1.3%, 1.3%, 2.4%, 2.2%, 1.5%, and 1.3% norleucine misincorporation in the six methionine-containing tryptic peptides obtained from the recombinant protein produced using AF3 process performed without met feed using the 64B4 host. (See Table 9.) However, no norleucine was detected in the recombinant proteins purified from AF2 and AF3 fermintation processes using the 66F8 *metA*(Y294C) host and 64B4 *metA*(Y294C) hosts, respectively. (See Tables 8 and 9 above.)

The tryptic peptide map analyses indicated that the recombinant protein pools purified from the mutant host cell fermentations contained less than detectable levels of norleucine misincorporation, while the control host cell fermentation performed without a methionine feed accumulated high levels of norleucine in the methionine-containing peptides. These results showed that use of *E. coli* host cell strains of the present invention resulted in the reduction or prevention of norleucine incorporation into heterologous (*e.g.*, recombinant) polypeptides.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

CLAIMS

What is claimed is:

1. A method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

2. A method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

3. A method for preventing or reducing norleucine misincorporation into a protein or a polypeptide, the method comprising expressing the protein or the polypeptide in an *E. coli*, wherein the microorganism comprises a mutant *metA* allele and a mutant *metK* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, and further wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

4. A method for producing a protein or a polypeptide in an *E. coli* host cell, wherein the protein or the polypeptide is free of norleucine misincorporation, the method comprising expressing in the *E. coli* host cell a nucleic acid encoding the protein or the polypeptide under culture conditions suitable to allow for expression of the protein or the polypepetide, wherein the *E. coli* host cell comprises a mutant *metA* allele, wherein the mutant *metA* allele encodes for MetA having an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26.

5. A method for producing a protein or a polypeptide in an *E. coli* host cell, wherein the protein or the polypeptide is free of norleucine misincorporation, the method comprising expressing in the *E. coli* host cell a nucleic acid encoding the protein or the polypeptide under culture conditions suitable to allow for expression of the protein or the polypepetide, wherein the *E. coli* host cell comprises a mutant *metK* allele, wherein the mutant *metK* allele encodes for MetK having an amino acid sequence encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28.

6. The method of claim 4 or claim 5, wherein the protein or the polypeptide is an antibody or an antibody fragment.

7. The method of claim 4 or claim 5, wherein the antibody or the antibody fragment is an anti-VEGF antibody or an anti-VEGF antibody fragment.

8. The method of claim 4 or claim 5, wherein the protein or the polypeptide is an antibody or an antibody fragment selected from the group consisting of an anti-VEGF antibody or an anti-VEGF antibody fragment, and wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:46 and nucleic acid encoding the amino acid sequence of SEQ ID NO:47.

9. The method of claim 4 or claim 5, wherein the protein or the polypeptide is an antibody or an antibody fragment selected from the group consisting of an anti-VEGF antibody or an anti-VEGF antibody fragment, and wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of the nucleic acid sequence comprising SEQ ID NO: 33 and the nucleic acid sequence comprising SEQ ID NO:34.

10. The method of claim 4 or claim 5, wherein the antibody or the antibody fragment is an anti-Factor D antibody or an anti-Factor D antibody fragment.

11. The method of claim 4 or claim 5, wherein the protein or the polypeptide is an antibody or an antibody fragment selected from the group consisting of an anti-Factor D antibody and an anti-Factor D antibody fragment, and wherein nucleic acid encoding the anti-

Factor D antibody or the anti-Factor D antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:48 and nucleic acid encoding the amino acid sequence of SEQ ID NO:49.

12. The method of claim 4 or claim 5, wherein the antibody or the antibody fragment is an anti-MET antibody or an anti-MET antibody fragment.

13. The method of claim 4 or claim 5, wherein the protein or the polypeptide is an antibody or an antibody fragment selected from the group consisting of an anti-MET antibody and an anti-MET antibody fragment, and wherein nucleic acid encoding the anti-MET antibody or the anti-MET antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:50, nucleic acid encoding the amino acid sequence of SEQ ID NO:51, and nucleic acid encoding the amino acid sequence of SEQ ID NO:52.

14. The method of claim 4 or claim 5, wherein expressing the protein or the polypeptide in the *E. coli* host cell is performed in the absence of exogenously added methionine to the culture medium or without a methionine feed.

15. An *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further

comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment.

16. The *E. coli* host cell of claim 15, wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of nucleic acid sequence comprising SEQ ID NO: 33 and nucleic acid sequence comprising SEQ ID NO:34.

17. The *E. coli* host cell of claim 15, wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:46 and nucleic acid encoding the amino acid sequence of SEQ ID NO:47.

18. An *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-VEGF antibody or an anti-VEGF antibody fragment.

19. The *E. coli* host cell of claim 18, wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of the nucleic acid sequence comprising SEQ ID NO: 33 and nucleic acid sequence comprising SEQ ID NO:34.

20. The *E. coli* host cell of claim 18, wherein nucleic acid encoding the anti-VEGF antibody or the anti-VEGF antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:46 and nucleic acid encoding the amino acid sequence of SEQ ID NO:47.

21. An *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment.

22. The *E. coli* host cell of claim 21, wherein nucleic acid encoding the anti-Factor D antibody or the anti-Factor D antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:48 and nucleic acid encoding the amino acid sequence of SEQ ID NO:49.

23. An *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-Factor D antibody or an anti-Factor D antibody fragment.

24. The *E. coli* host cell of claim 23, wherein nucleic acid encoding the anti-Factor D antibody or the anti-Factor D antibody fragment is selected from the group consisting of

nucleic acid encoding the amino acid sequence of SEQ ID NO:48 and nucleic acid encoding the amino acid sequence of SEQ ID NO:49.

25. An *E. coli* host cell comprising a mutant *metA* allele, wherein the mutant *metA* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment.

26. The *E. coli* host cell of claim 25, wherein nucleic acid encoding the anti-MET antibody or the anti-MET antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:50, nucleic acid encoding the amino acid sequence of SEQ ID NO:51, and nucleic acid encoding the amino acid sequence of SEQ ID NO:52.

27. An *E. coli* host cell comprising a mutant *metK* allele, wherein the mutant *metK* allele comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:28, wherein the *E. coli* host cell further comprises nucleic acid encoding an anti-MET antibody or an anti-MET antibody fragment.

28. The *E. coli* host cell of claim 27, wherein nucleic acid encoding the anti-MET antibody or the anti-MET antibody fragment is selected from the group consisting of nucleic acid encoding the amino acid sequence of SEQ ID NO:50, nucleic acid encoding the amino

acid sequence of SEQ ID NO:51, and nucleic acid encoding the amino acid sequence of SEQ ID NO:52.