PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing (*day/month/year*)	19 OCT 2006
Applicant's or agent's file reference 10498-00093	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US05/41953	16 November 2005 (16.11.2005)	16 November 2004 (16.11.2004)

International Patent Classification (IPC) or both national classification and IPC

IPC(8): **C12N 15/74**(2006.01)
USPC: 435/471,477,481

Applicant

PRESIDENT AND FELLOWS OF HARVARD COLLEGE

1. This opinion contains indications relating to the following items:

- [X] Box No. I — Basis of the opinion
- [ ] Box No. II — Priority
- [ ] Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV — Lack of unity of invention
- [X] Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI — Certain documents cited
- [ ] Box No. VII — Certain defects in the international application
- [ ] Box No. VIII — Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis(b)* that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	29 September 2006 (29.09.2006)	Jennifer Dunston Telephone No. 571-272-0507

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/41953

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☐ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☐ on paper

☐ in electronic form

c. time of filing/furnishing

☐ contained in the international application as filed.

☐ filed together with the international application in electronic form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/41953

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims 3 and 13-16	YES
	Claims 1, 2, 4-12 and 17-22	NO
Inventive step (IS)	Claims NONE	YES
	Claims 1-22	NO
Industrial applicability (IA)	Claims 1-22	YES
	Claims NONE	NO

2. Citations and explanations:

Please See Continuation Sheet

Supplemental Box
In case **the space in any of the preceding boxes is not sufficient.**

V. 2. Citations and Explanations:
Claims 1, 2, 4-12 and 18-22 lack novelty under PCT Article 33(2) as being anticipated by Kiel et al.
Regarding claims 1 and 22, Kiel et al teach a method of consecutive integration of single copies of a heterologous gene at multiple locations in the *Bacillus subtilis* chromosome by replacement recombination, comprising the steps of (a) contacting a sample of *Bacillus subtilis* cells with a first exogneous nucleic acid sequence having a thyP3 selection marker, and allowing the nucleic acid sequence to exchange with a nucleic acid sequence in the cells to produce first recombinant target cells, (b) contacting the cells resulting from step (a) with a minimal medium lacking thymine and thymidine to select for prototrophs, (c) contacting the cells resulting from step (b) with a second exogenous nucleic acid sequence, and allowing the second exgoneous nucleic acid sequence to exchange with the first nucleic acid sequence, and (d) contacting the cells resulting from step (c) with a selection substrate comprising timethoprim (TMP), thereby providing a sample of further recombinant cells (e.g. pages 4244-4246, Materials and Methods; pages 4246-4248, Results; Figure 1).
Regarding claim 2, the selection marker of Kiel et al comprises a single selection component, thyP3 (e.g. page 4246, right column).
Regarding claim 4, the thyP3 selection marker of Kiel et al functions as a positive and negative selection component (e.g. page 4246, right column).
Regarding claim 5, the thyP3 selection marker of Kiel et al provides growth in media lacking thymine and thymidine when present (e.g. page 4246, right column).
Regarding claim 6, the thyP3 selection marker is lethal to the cell in the presence of TMP (e.g. page 4248, left column).
Regarding claim 7, the method of Kiel et al uses a minimal medium for the first selection (e.g. page 4244, right column, last full paragraph; page 4246, right column).
Regarding claim 8, the genome of the cells is altered by the addition of more than one nucleotide not previously present (e.g. Figure 1).
Regarding claim 9, the genome of the cells is altered by the removal of more than one nucleotide previously present (e.g. Figure 1).
Regarding claim 10, the genome of the cells is altered by the exchange of more than one nucleotide (e.g. Figure 1).
Regarding claim 11, the genome of the cells is altered by the removal of amyE, addition of thyP3 and exchange of thyP3 for glgB (e.g. Figure 1).
Regarding claim 12, the endogenous amyE gene is altered (e.g. Figure 1).
Regarding claim 17, the alterations made to the genome of the cell alter the nucleic acid sequence and will necessarily alter the restriction enzyme sites present in that region of the genome (e.g. Figure 1).
Regarding claim 18, the *Bacillus subtilis* cell of Kiel et al is a bacterial cell.
Regarding claim 19, Kiel et al teach a method of consecutive integration of single copies of a heterologous gene at multiple locations in the *Bacillus subtilis* chromosome by replacement recombination, comprising the steps of (a) contacting a sample of *Bacillus subtilis* cells with a first exogneous nucleic acid sequence having a thyP3 selection marker and an Em^r gene, and allowing the nucleic acid sequence to

Supplemental Box
In case **the space in any of the preceding boxes is not sufficient.**

exchange with a nucleic acid sequence in the cells to produce first recombinant target cells, (b) contacting the cells resulting from step (a) with a minimal medium lacking thymine and thymidine to select for prototrophs, (c) contacting the cells resulting from step (b) with a second exogenous nucleic acid sequence, and allowing the second exgoneous nucleic acid sequence to exchange with the first nucleic acid sequence, and (d) contacting the cells resulting from step (c) with a selection substrate comprising timethoprim (TMP), thereby providing a sample of further recombinant cells (e.g. pages 4244-4246, Materials and Methods; pages 4246-4248, Results; Figure 1). Regarding claims 20 and 21, Kiel et al teach a method of consecutive integration of single copies of a heterologous gene at multiple locations in the *Bacillus subtilis* chromosome by replacement recombination, comprising the steps of (a) contacting a sample of *Bacillus subtilis* cells with a first exogneous nucleic acid sequence having a thyP3 selection marker and an Em^r gene, and allowing the nucleic acid sequence to exchange with a nucleic acid sequence in the cells to produce first recombinant target cells, (b) contacting the cells resulting from step (a) with a minimal medium lacking thymine and thymidine to select for thymine-prototrophs and Em^r (e.g. pages 4244-4246, Materials and Methods; pages 4246-4248, Results; Figure 1).

Claim 3 lacks an inventive step under PCT Article 33(3) as being obvious over Kiel et al. The teachings of Kiel et al are described above and applied as before. Further, Kiel et al teach that the thyP3 gene is closely related to the endogenous thyA gene (e.g. pages 4246-4247, Results). Kiel et al teach that the thyP3 gene can recombine with the mutant thyA gene present in the strain (e.g. pages 4246-4247, Results). It would have been obvious at the time the invention was made for one to use the method of Kiel et al to remove the thyA gene from the strain and to use thyA as the selection marker. One would have been motivated to make such a modification to prevent any unwanted recombination between thyP2 and the endogenous mutant thyA and to use the thyA gene, which is normally expressed in that species and is necessarily adapted to function in that strain.

Claims 13-16 lack an inventive step under PCT Article 33(3) as being obvious over Kiel et al in view of Doring et al. Kiel et al teach a method of consecutive integration of single copies of a heterologous gene at multiple locations in the *Bacillus subtilis* chromosome by replacement recombination, comprising the steps of (a) contacting a sample of *Bacillus subtilis* cells with a first exogneous nucleic acid sequence having a thyP3 selection marker, and allowing the nucleic acid sequence to exchange with a nucleic acid sequence in the cells to produce first recombinant target cells, (b) contacting the cells resulting from step (a) with a minimal medium lacking thymine and thymidine to select for prototrophs, (c) contacting the cells resulting from step (b) with a second exogenous nucleic acid sequence, and allowing the second exgoneous nucleic acid sequence to exchange with the first nucleic acid sequence, and (d) contacting the cells resulting from step (c) with a selection substrate comprising timethoprim (TMP), thereby providing a sample of further recombinant cells (e.g. pages 4244-4246, Materials and Methods; pages 4246-4248, Results; Figure 1). Kiel et al do not teach the introduction of exogenous tRNA synthetase genes with altered amino acid specificity into the bacteria. Doring et al teach the introduction of mtated aminoacyl tRNA synthetases having their editing site mutated and capable of mischarging cogante tRNA with a noncanonical amino acid to diversify the produciton of proteins in a microbial cell (e.g. Abstract; paragraphs [0051]-[0061]. Doring et al teach the use of cells capable of homologous recombination (e.g. paragraphs [0060]-[0062]). It would have been obvious to use the method of Kiel et al to introduce the mutated tRNA synthetase of Doring et al, because Doring et al teach the introduction of the tRNA synthetase gene by homologous recombination and Kiel et al teach a method of homolgous recombination to introduce an exogenous gene. One would have been motivated to make such a modification to be able to increase protein diversity in the *Bacillus subtilis* cells.

Claims 1-22 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.