Hence, there is a need for efficient replication of vectors comprising homopolymeric sequences with high fidelity.

Summary

A solution to these problems is the provision of covalently closed circular recombinant DNA molecules in which the homopolymeric sequence is not located directly next to the origin of replication in the direction of replication, but in which there is a certain distance between the homopolymeric sequence and the origin or replication.

In one aspect, the present invention refers to covalently closed circular recombinant DNA molecule comprising: a bacterial origin of replication, an insert comprising a homopolymeric region, wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication.

Accordingly, disclosed herein is a covalently closed circular recombinant DNA molecule comprising:

- an origin of replication,
- an insert comprising a homopolymeric region,

wherein the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication.

Also disclosed herein is a covalently closed circular recombinant DNA molecule comprising:

- an origin of replication,
- an insert comprising a homopolymeric region,

wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication.

The covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication may improve the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular

recombinant DNA molecule in which the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication.

Further, the covalently closed circular recombinant DNA molecule in which the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication improves the yield of the covalently closed circular recombinant DNA molecule in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication.

The covalently closed circular recombinant DNA molecule may be a plasmid, cosmid, bacterial artificial chromosome (BAC), bacteriophage, viral vector or hybrids thereof. Preferably, the covalently closed circular recombinant DNA molecule is a plasmid.

Typically, the origin of replication is of bacterial origin. Preferably, the origin of replication is a high copy number origin. The origin of replication may be derived from the pBR322 plasmid, pUC plasmid, pMB1 plasmid, ColE1 plasmid, R6K plasmid, p15A plasmid, pSC101 plasmid or F1 phagemid. Preferably, the origin of replication is derived from the pUC plasmid.

In some examples, the covalently closed circular recombinant DNA molecule further comprises a primosome assembly site in the heavy strand (PAS-BH).

Typically, the covalently closed circular recombinant DNA molecule further comprises a selection marker, for example an antibiotic resistance gene or a sucrose selectable marker.

In preferred examples the homopolymeric region comprised in the insert of the covalently closed circular recombinant DNA molecule comprises a poly(A) sequence and/or a poly(C) sequence. In more preferred examples the homopolymeric region comprises a poly(C) sequence. In even more preferred examples the homopolymeric region comprises a poly(C) and a poly(A) sequence. Preferably, the poly(A) sequence comprises a sequence of about 20 to about 400 adenosine nucleotides more preferably of about 60 to about 250 adenosine nucleotides. Preferably, the poly(C) sequence comprises a sequence of about 10 to 200 cytidine nucleotides, more preferably of about 20 to 40 cytidine nucleotides. In particularly preferred examples the homopolymeric region comprises a poly(A) sequence of about 60 to about 70 adenosine nucleotides and a poly(C) sequence of about 20 to 30 cytidine nucleotides.

Also described herein is the use of a covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of least 500 bp from the origin of

replication in the direction of replication for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication.

Further described herein is a covalently closed circular recombinant DNA molecule in which the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication.

When the closed circular recombinant DNA molecule is used in which the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication, the fermentation time may be shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication.

When the closed circular recombinant DNA molecule is used in which the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication, the fermentation time is shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication.

Also disclosed herein is a method for fermentative production of a covalently closed recombinant DNA molecule comprising the steps of:

(a) providing a microorganism comprising the covalently closed circular recombinant DNA molecule as disclosed herein;

(b) fermenting the microorganism of step (a).

In a specific example, the method further comprises the following step:

(c) isolating the covalently closed circular recombinant DNA molecule from the microorganism of step(b).

In particularly preferred examples the microorganism is a bacterium, preferably E. coli. In preferred examples the microorganism is a bacterium (e.g. E. coli) containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid and step (b) comprises the following steps:

(i) growing the bacteria containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid at a temperature in the range of 25°C to 32°C during the growth phase of the fed-batch phase wherein the substrate is supplied at a rate such that the growth rate is μ=0.05 to 0.3hr^{-1} during the fed-batch phase,

(ii) inducing production of said DNA plasmid after the growth phase by increasing the temperature to 36°C to 45°C; and

(iii) continuing fermentation at the temperature applied (ii) to accumulate the said DNA plasmid.

In a more specific example , the temperature applied in step (i) is about 30°C and the temperature applied in step (ii) is about 42°C.

In the above described methods, the yield of the covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of least 500 bp from the origin of replication in the direction of replication is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication.

The yield of the covalently closed circular recombinant DNA in which the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the

same as the direction of replication of the origin of replication is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication. Moreover, the fermentation time is shortened, compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication.

Further disclosed herein is a method that the sequence of the covalently closed circular recombinant DNA molecule remains stable during fermentative production.
For example, the homopolymeric region remains stable during fermentative production.
In another aspect, the present invention refers to the use of a covalently closed circular recombinant DNA molecule as disclosed herein for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication. In a further aspect, the present invention refers to the use of a covalently closed circular recombinant DNA molecule disclosed herein for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

In a further aspect, there is disclosed a method for fermentative production of a covalently closed recombinant DNA molecule comprising the steps of (a) providing a microorganism comprising the covalently closed circular recombinant DNA molecule as disclosed herein; (b) fermenting the microorganism of step (a).

In one aspect, the present invention refers to a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps: (a) providing a covalently closed circular recombinant DNA molecule having a bacterial origin

of replication, and an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication; (b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region sequence is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication.

In yet another aspect, the present invention refers to a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps: (a) providing a covalently closed circular recombinant DNA molecule having a bacterial origin of replication, and an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the insert comprising the homopolymeric region sequence is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, and wherein the homopolymeric region is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication; (b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.
In a further aspect, the present invention refers to the use of the covalently closed circular recombinant DNA molecule disclosed herein for the *in vitro* transcription of RNA.

Disclosed herein is a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having
- an origin of replication, and
- an insert comprising a homopolymeric region, wherein the homopolymeric region is located at a distance of less than 500 bp from the origin of replication in the direction of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the homopolymeric region is located at a distance of at least 500 bp from the origin of replication in the direction of replication.

Also disclosed herein is a method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having
- an origin of replication, and
- an insert comprising a homopolymeric region, wherein the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the origin of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the origin of replication.

Finally, also described herein is the use of the covalently closed circular recombinant DNA molecule as disclosed herein for in vitro transcription of RNA.

Brief description of the figures

Figure 1: Vector map of P1140

The orientation of P1140 is such that the direction of replication of the origin of replication (denoted in the vector map as pUC origin) is opposite to the direction of transcription of the insert (denoted in the vector map as RNA).

Figure 2: Vector map of P1140-AF2

The orientation of P1140-AF2 is such that the direction of replication of the origin of replication (denoted in the vector map as pUC origin) is the same as the direction of transcription of the insert (RNA denoted in the vector map as RNA).

Figure 3: Vector map of P1140-AF1

The orientation of P1140-AF1 is such that the direction of replication of the origin of replication (denoted in the vector map as pUC origin) is opposite to the direction of transcription of the insert (denoted in the vector map as RNA).

Figure 4: Vector map of P1140-K2

CLAIMS

1. Covalently closed circular recombinant DNA molecule comprising:
- a bacterial origin of replication,
- an insert comprising a homopolymeric region,
wherein the homopolymeric region comprises at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides and is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication.

2. Covalently closed circular recombinant DNA molecule of claim 1, wherein the homopolymeric region comprises at least one poly(C) sequence and/or at least one poly(A) sequence.

3. Covalently closed circular recombinant DNA molecule of claim 1 or 2, wherein the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.

4. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the yield of the covalently closed circular recombinant DNA molecule in fermentative production is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.

5. Covalently closed circular recombinant DNA molecule according to claim 3, wherein the yield of the covalently closed circular recombinant DNA molecule in fermentative production is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the

homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

6. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule is selected from the group consisting of plasmid, cosmid, bacterial artificial chromosome (BAC), bacteriophage, viral vector or hybrids thereof.

7. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule is a plasmid.

8. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is a high copy number origin.

9. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is derived from the pBR322 plasmid, pUC plasmid, pMB1 plasmid, ColE1 plasmid, R6K plasmid, p15A plasmid, or pSC101 plasmid.

10. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the bacterial origin of replication is derived from the pUC plasmid.

11. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule further comprises a primosome assembly site in the heavy strand (PAS-BH).

12. Covalently closed circular recombinant DNA molecule according to any one of the previous claims, wherein the covalently closed circular recombinant DNA molecule further comprises a selection marker.

13. Covalently closed circular recombinant DNA molecule according to claim 11, wherein the selection marker is an antibiotic resistance gene.

14. Covalently closed circular recombinant DNA molecule according to claim 11, wherein the selection marker is a sucrose selectable marker.

15. Covalently closed circular recombinant DNA molecule according to claim 7, wherein the plasmid is derived from the backbone having the sequence identified in SEQ ID NO: 4 or having the sequence identified in SEQ ID NO: 5.

16. Covalently closed circular recombinant DNA molecule according to claim 2, wherein the poly(A) sequence comprises a sequence of about 20 to about 400 adenosine nucleotides.

17. Covalently closed circular recombinant DNA molecule according to claim 16, wherein the poly(A) sequence comprises a sequence of about 60 to about 250 adenosine nucleotides.

18. Covalently closed circular recombinant DNA molecule according to claims 15 to 17, wherein the poly(C) sequence comprises a sequence of about 15 to 200 cytidine nucleotides.

19. Covalently closed circular recombinant DNA molecule according to claim 18, wherein the poly(C) sequence comprises a sequence of about 20 to 40 cytidine nucleotides.

20. Use of a covalently closed circular recombinant DNA molecule, according to any one of claims 1 to 19 for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.

21. Use of a covalently closed circular recombinant DNA molecule, according to any one of claims 2 and 4 to 19 for increasing the yield of the covalently closed circular recombinant DNA in fermentative production compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

22. Use according to claim 20, wherein the fermentation time is shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.

23. Use according to claim 21, wherein the fermentation time is shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

24. Method for fermentative production of a covalently closed recombinant DNA molecule comprising the steps of:

(a) providing a microorganism comprising the covalently closed circular recombinant DNA molecule according to claims 1 to 19;

(b) fermenting the microorganism of step (a).

25. The method according to claim 24, further comprising a step of

(c) isolating the covalently closed circular recombinant DNA molecule from the microorganism of step(b).

26. Method of claim 24 or 25, wherein the microorganism is a bacterium containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid and step (b) comprises the following steps:

(i) growing the bacteria containing a covalently closed circular recombinant temperature inducible high copy DNA plasmid at a temperature in the range of 25°C to 32°C during the growth phase of the fed-batch phase wherein the substrate is supplied at a rate such that the growth rate is μ=0.05 to 0.3hr^{-1} during the fed-batch phase,

(ii) inducing production of said DNA plasmid after the growth phase by increasing the temperature to 36°C to 45°C; and

(iii) continuing fermentation at the temperature applied (ii) to accumulate the said DNA plasmid.

27. Method according to claim 26, wherein the temperature applied in step (i) is about 30°C and the temperature applied in step (ii) is about 42°C.

28. Method according to any one of claims 24 to 27, wherein the yield of the covalently closed circular recombinant DNA molecule according to claims 1 to 19 is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.

29. Method according to any one of claims 24 to 27, wherein the yield of the covalently closed circular recombinant DNA molecule according to claims 1 to 21 is increased compared to the yield of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

30. Method according to claim 28, wherein the fermentation time is shortened, compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication.

31. Method according to claim 29, wherein the fermentation time is shortened compared to the fermentation time of a covalently closed circular recombinant DNA molecule in which the insert comprising the homopolymeric region is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication.

32. Method according to any one of claims 24 to 31, wherein the homopolymeric region sequence remains stable during the fermentation production.

33. Method according to any one of claims 24 to 32, wherein the sequence of the covalently closed circular recombinant DNA molecule remains stable during fermentation production.

34. Method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having

- a bacterial origin of replication, and
- an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the homopolymeric region is located at a distance of less than 500 bp from the bacterial origin of replication in the direction of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region sequence is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication.

35. Method for improving the yield of a covalently closed circular recombinant DNA molecule comprising the following steps:

(a) providing a covalently closed circular recombinant DNA molecule having
- a bacterial origin of replication, and
- an insert comprising a homopolymeric region comprising at least one homopolymeric sequence comprising a stretch of at least 15 identical nucleotides, wherein the insert comprising the homopolymeric region sequence is oriented so that the direction of transcription of the insert is opposite to the direction of replication of the bacterial origin of replication, and wherein the homopolymeric region is located at a distance of at least 500 bp from the bacterial origin of replication in the direction of replication;

(b) modifying the covalently closed circular recombinant DNA molecule of (a) so that the insert comprising a homopolymeric region is oriented so that the direction of transcription of the insert is the same as the direction of replication of the bacterial origin of replication.

36. Use of the covalently closed circular recombinant DNA molecule according to claims 1 to 19 for the *in vitro* transcription of RNA.