Automation-ready dna cloning by bacterial natural transformation

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(54) **Title:** AUTOMATION-READY DNA CLONING BY BACTERIAL NATURAL TRANSFORMATION

(57) Abstract: The present invention provides a simple and efficient method of cloning and preparing circular plasmids using Acinetobacter natural transformation. The circular plasmids carrying target DNA molecules are prepared by transforming Acinetobacter with linear DNA multimers.

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TITLE

Automation-ready DNA cloning by bacterial natural transformation

FIELD OF THE INVENTION

The present invention concerns the field of cloning and preparing of plasmids. It uses Acinetobacter natural transformation for high efficiency preparation of circular plasmid carrying target DNA, by simply adding linear DNA multimers to cells of Acinetobacter.

BACKGROUND OF THE INVENTION

Cloning of target DNA molecules and preparation of recombinant plasmids has traditionally been performed using E. coli.

E. coli cells need to be made competent or permeable and then artificially transformed by either heat shock or electroporation in order for the cells to take up DNA. To create competent cells for either transformation method, the bacterial cells are grown to logarithmic phase and harvested, since exponential-phase cells can be rendered competent more easily than cells at other stages of growth. Preparation of chemically competent cells involves a series of cold salt washes to disrupt the cell membranes, preparing the cells to accept DNA; while for preparation of electro-competent cells, the cells are chilled and washed with a series of cold deionized water and 10% glycerol (a low-salt environment is important when electrical currents are involved).

To introduce the desired DNA molecules into chemically competent cells, the DNA is mixed with the chilled cells and incubated on ice to allow the DNA to come into close contact with the cells. The DNA-cell mixture is then briefly heated to 42-50°C, allowing the DNA to enter the cell through the disrupted membrane. The heated mixture is then placed back on ice to retain the DNA inside the bacteria. Many cells do not survive the rapid temperature change but enough maintain integrity to retain the DNA and, when growth medium is added, recover and divide. In the whole process, temperature needs to be precisely controlled. Any warm-up of the cells before the heat shock results in loss of competence.

When electroporation is used to introduce DNA molecules into electro-competent cells, the cells are also incubated on ice with the DNA. However, the DNA-cell mixture is then exposed to an electrical current, thereby opening pores in the cell membrane so that the DNA can enter the cell. Some cells do not survive this treatment but many are able to replicate once growth medium is added. If the DNA solution has too much salt in it, arcing can occur, compromising the transformation.

Preparation and transformation of chemically and electro-competent E.coli cells is tedious and un-suited for robotics.

The present invention addresses and overcomes the limitations set out above and provides a method of cloning and preparing circular plasmids suitable for robotics and automation. The method is particularly relevant for plasmids intended for later use in gram negative bacteria.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method for preparing circular plasmid DNA, said method comprising the steps of:

a) providing a linear DNA multimer comprising a target DNA molecule and a linearized plasmid backbone DNA molecule, wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least two,
b) contacting Acinetobacter cells with said linear DNA multimer,
c) recovering circularized plasmid DNA from naturally transformed Acinetobacter cells obtained from step (b), wherein said circularized plasmid DNA comprises said target DNA molecule and said linearized plasmid backbone DNA molecule.

In a second aspect, the invention concerns the use of Acinetobacter cells for preparing circular plasmid DNA from an extracellular linear DNA multimer, wherein said linear DNA multimer comprises a target DNA molecule and a linearized plasmid backbone DNA molecule, and wherein the copy number of

said target DNA molecule and/or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least two.

In a third aspect, the invention provides an Acinetobacter cell comprising a circularized plasmid, wherein said circularized plasmid comprises an exogenous target DNA molecule and a linearized plasmid backbone DNA molecule.

DESCRIPTION OF THE INVENTION

Description of the figures:

Figure 1: A cartoon showing the RppA gene cluster from Streptomyces coelicolor A3(2); the genomic DNA fragment to be amplified, and the respective amplified DNA fragments for cloning in Acinetobacter.

Figure 2: Image of an agarose gel following electrophoretic separation of linearized plasmid backbone DNA molecule (derived from pUPC24), target DNA molecules (derived from RppA cluster of Streptomyces), and a DNA multimer comprising the backbone and targets. Lane 1) lkb ladder, 2) linearized plasmid backbone (BB) PCR fragment, 3) 8.1 K = PCR fragment of first half of 8kb RppA target DNA molecule, 4) 8.2K = PCR fragment of second half of 8kb RppA target DNA molecule, 5) DNA multimer prepared by Gibson reaction, 6) blank, 7) DNA multimer prepared by POE-PCR method, 8) lkb ladder.

Figure 3: Image of an agarose gel following electrophoretic separation of PCR-amplified target DNA molecules derived from Acinetobacter cells transformed with linear DNA multimers comprising RppA DNA. PCR amplified RppA fragment from positive control plasmid shows the expected molecular weight.

Figure 4: Image of colonies on LB plates plated with Acinetobacter, E. coli, and B. subtilis cells transformed with linear DNA multimer comprising pXJDP linearized plasmid backbone DNA molecule and cmr-plac-gft target DNA molecule.

Abbreviations, terms, and definitions:

Automated: means operated largely by automatic equipment, such as using a machine and/or computer (i.e. a robot) to perform a process in order to reduce the amount of work done by humans and the time taken to do the work.

DNA multimer: refers to a linear DNA molecule comprising a target DNA molecule and a linearized plasmid backbone DNA molecule, wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule is at least two.

Linearized plasmid backbone DNA molecule: refers to a DNA molecule comprising an origin of replication (ORI) and optionally other standard plasmid elements such a means for selection (for example a gene encoding a selecting marker such as antibiotic resistance) of host cells harboring the plasmid.

Natural transformation: a bacterial mechanism for actively taking up DNA from the environment. Bacteria capable of natural transformation do not need to be made competent by artificial means such as any chemical or electrochemical means recognized by a person skilled in the art.

Target DNA molecule: can be any natural or synthetic DNA molecule, e.g. a gene and/or an operon of interest with or without regulatory sequences for expression in a host cell.

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

GenBank accession#: GenBank accession number groups all gi numbers for a specific sequence into an ordered series. The GenBank accession number consists of a base Accession number, a dot, and a version suffix that starts with 1. The base Accession number identifies the sequence record, and the version suffixes form the series of versions, starting with 1. A sequence Accession number without a version suffix always refers to the latest version of the sequence.

Detailed description of the invention:

A fast and automated cloning platform is urgently needed to match the rapid development of DNA sequencing and genome mining. As already mentioned, existing methods for cloning and preparation of plasmids in E. coli are

cumbersome and time-consuming, and not easily adaptable for automation and robotics. These existing cloning methods mainly rely on electroporation of E. coli competent cells with purified DNA substrate, and further require intensive colony PCR screening due to the low yield of positive clones comprising target DNA molecules, especially when the target DNA molecules are large. All this makes it difficult to perform the cloning and plasmid preparation on a high throughput scale. To prepare E. coli competent cells (chemical as well as electro-competent), the cells need to be washed extensively, such as by centrifugation and buffer exchange at controlled temperature. Implementing such protocols on lab-automation hardware requires sophisticated and expensive hardware. While robots handle pipetting well, they are much less efficient in DNA purification and bacterial colony picking. As a result, cloning of e.g. large target DNA molecules or complex refactoring of gene clusters is challenging for those robots.

In comparison to classical transformation using chemical competent cells or electroporation, the present invention provides a highly simplified method for cloning and preparing plasmids using the naturally competent bacterium, Acinetobacter. Surprisingly, Acinetobacter is highly efficiency in generating circular plasmid comprising target DNA molecules, by the simple addition of linear DNA multimers (e.g. products generated by Gibson reaction or overlap extension PCR) to the Acinetobacter culture.

Natural transformation is a sophisticated mechanism found in specific bacteria that actively take up DNA from the environment. The capacity for natural transformation appears to occur in several different prokaryotic species in different phyla. During the process, competence pili reach out from cells and catch DNA molecules by their tips, and pull them back to the cell surface. An ATP-dependent protein complex digests one of the DNA strands while transporting the other strand across the cell membrane into the cytoplasm where the single DNA strand is then incorporated into the chromosome by homologous recombination. Normally bacteria use this mechanism to acquire DNA from sister cells for use as a chromosome repair template or to get new genetic traits from related species.

Acinetobacter baylyi ADP1, a non-pathogenic gram-negative bacterium from the class Pseudomonadales, is a model organism for molecular biology. It is known to grow fast on simple media with versatile carbon sources. Its robust metabolism and high adaptation capacity are also attractive characteristics for biotechnological and environmental applications.

A. baylyi ADP1 has previously been used to study horizontal gene transfer in situ [Nielsen et al 1997], and the dissemination and persistence of plant transgenes in soil [Metzgar et al 2004], to construct single gene knockout library [Durot et al 2008], to improve biochemical or enzyme production by metabolic engineering [Metzgar et al 2004], and to do multiplex genomic engineering [West et al 1994]. While these prior art studies take advantage of the natural transformation capability of Acinetobacter, they all depend on the incorporation of the transformed DNA into the chromosome of *A. baylyi* ADP1 by homologous recombination. In fact, most prior art related to Acinetobacter concerns direct incorporation of exogenous DNA taken up by Acinetobacter by natural transformation into the chromosome by homologous recombination events. Further, one study shows efficient replication and maintenance of a shuttle plasmid in Acinetobacter, as a tool for gene cloning and expression in Acinetobacter [Lucidi et al 2018]. However, it is noted that this shuttle plasmid was first constructed in E.coli using traditional methods; it was then purified from the E.coli, and used to transform Acinetobacter by natural transformation. As evident from example 5 herein, this process (natural transformation with circular plasmid DNA) has a very low efficiency, and further requires large amounts of purified circular plasmid DNA.

The use of Acinetobacter for direct cloning and preparation of plasmids, however, has not previously been reported. The present invention exploits the unexpected ability of Acinetobacter to circularize linear multimeric DNA copies upon uptake resulting in plasmid formation, rather than chromosomal incorporation. The method of cloning and preparing plasmids relies on transforming Acinetobacter with linear DNA multimers comprising multiple copies of a linearized plasmid backbone DNA molecule as well as one or more target DNA molecules of interest. The linear DNA multimer is taken up by Acinetobacter by natural transformation, and the cell processes it into at least

one plasmid copy. For the efficient processing of a linear DNA construct into a plasmid, the linear DNA construct provided to the Acinetobacter cell must be a multimer - i.e. the copy number of the target DNA molecule and/or the backbone DNA molecule of the linear DNA construct must be at least two. When the linearized plasmid backbone DNA molecule harbors an origin of replication that is functional in Acinetobacter, the plasmid is replicated within the cell; and plasmids can be extracted by standard plasmid purification protocols.

Other microorganisms are known to be capable of natural transformation, such as *Bacillus subtilis.* Transformation of DNA multimer in B. subtilis for obtaining chimeric plasmids has been reported [You et al 2011]. However, the present invention of preparing plasmids by Acinetobacter natural transformation provides significant improvements over other bacterial hosts for the following reasons:

Firstly, Acinetobacter (*Acinetobacter calcoaceticus* BD413 now named *Acinetobacter baylyi* BD413) is 100 times more efficient in natural transformation, based on chromosomal integration of extracellular DNA, than wild type strains of Bacillus subtilis [Melnikov et al 1999]. Secondly, Acinetobacter exhibits essentially constitutive transformation competence in ordinary complex broth, such as LB, with no specific culture requirements. By contrast, a two-step growth in semi-synthetic media is required for optimal transformation of *B. subtilis.* Although super competent *B. subtilis* strains overexpressing the competence master regulator ComK have been engineered, these have the disadvantage of needing to add xylose to the medium for inducing competence [Zhang et al 2014].

Thirdly, plasmid recovery from Acinetobacter is easier/simpler than from Bacillus, and the widely available standard protocols and kits used for E. coli work equally efficiently for recovering plasmids from Acinetobacter. By contrast, the recovery of plasmids from Bacillus, a gram positive bacterium having a more resistant cell wall, requires an initial lysis treatment such as by use of the enzymes Lysozyme, Lysostaphin, Mutanolysin, or similar to break the peptidoglycan layers of the cell wall.

Finally, since Acinetobacter is a gram-negative bacterium, unlike Bacillus, the components of the linearized plasmid backbone DNA molecule required for self-replication and expression in Acinetobacter are directly compatible with those required for later replication and expression in e.g. E. coli and Pseudomonas putida (which are also gram negative bacterium). As a consequence, the size of the plasmid backbone, per se, can be kept at a minimum (which is often preferable), since it avoids duplications e.g. the need for two origins of replication; or gram strain specific selection markers. Elements required for conjugation may constitute yet other components relevant to have in a plasmid for engineering purposes. As gram positive and gram negative cells have a different cell membrane structure, the conjugation machinery of one would not function in the other.

In conclusion, Acinetobacter is a powerful platform for high through-put cloning and plasmid preparation, having several advantages over the prior art systems that rely on E. coli or Bacillus.

The examples provided with the present invention demonstrate that high efficiency cloning using Acinetobacter can be obtained for single genes as well as large gene clusters; even allowing multiple fragment assembly to be performed as part of the cloning procedure (see Examples 1 and 2). The method is especially favorable as it produces a high ratio of positive transformants as opposed to e.g. standard E. coli cloning systems where many transformants need to be screened as a high ratio of false-positives is often seen (see Examples 3 and 4).

I. A method for preparing circular plasmid

A first aspect of the present invention provides a method for preparing circular plasmid DNA, said method comprising the steps of:

a) providing a linear DNA multimer comprising a target DNA molecule and a linearized plasmid DNA backbone molecule, wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least two,

b) contacting Acinetobacter cells with said linear DNA multimer,

c) recovering circularized plasmid DNA from naturally transformed Acinetobacter cells obtained from step (b), wherein said circularized plasmid comprises a copy of said target DNA molecule and a copy of said linearized plasmid backbone DNA molecule.

The method for preparing circular plasmid DNA using Acinetobacter according to the first aspect of the invention may comprise an additional step (b') of:

(i) plating cells of Acinetobacter obtained from step (b) on solid medium, and

(ii) identifying Acinetobacter cell colonies harboring circularized plasmid, wherein said circularized plasmid DNA comprises said target DNA molecule and said linearized plasmid backbone DNA molecule;

wherein the Acinetobacter cells in step (c) are the cells identified in step (b') (ii).

The method of the present invention is preferably automated, such as by the use of robotics. Single selected steps or all steps may be carried out by automation using one or more robots. In one embodiment, selected steps of the method are carried out by use of robotics, such as in multiple well systems as recognized by a person skilled in the art. In a preferred embodiment, the steps of preparation of linear DNA multimer, transformation, as well as plasmid recovery are carried out by robotics. In a preferred embodiment, at least steps (b) and (c) described above are automated.

A target DNA molecule may be any natural or synthetic DNA. The target DNA molecule or a part thereof may for example comprise either a gene or an operon of interest for expression in a host.

In one embodiment, the target DNA molecule is 100-500 base pairs in size; in another embodiment, the target DNA molecule is 500bp-2kb; in yet another embodiment, the target DNA molecule is 2-10kb; in yet another embodiment, the target DNA molecule is more than lOkb in size.

A linearized plasmid backbone DNA molecule comprises an origin of replication in order for the final circularized plasmid product to be maintained by a host bacterium. Origin of replication (ORI) elements direct the host to use its cellular enzymes to make copies of the plasmid, and are thus essential for self-replication. Certain ORI have been discovered/engineered to maximize this replication, facilitating the production upwards of several hundred copies per bacterial cell. Most plasmids replicate only within a particular genus or family due to the specificity of the ORI; it is for example exceptional to find gram-positive plasmids that are able to replicate in E. coli.

In one embodiment, the linearized plasmid backbone DNA molecule comprises an origin of replication capable of replicating in Acinetobacter. Said origin of replication may be compatible with other host strains. In a preferred embodiment, the origin of replication is compatible with other gram-negative bacteria selected from the list E. coli, Pseudomonas putida, Klebsiella spp., Shewanella oneidensis, and Vibrio natriegens. In a most preferred embodiment, the origin of replication is compatible with E. coli.

In one embodiment, the linearized plasmid backbone DNA molecule comprises an origin of replication having the DNA sequence selected from the list SEQ ID NO.: 1, 2, 3, 4, 6, 7, 8, 9, 10, and 11.

In another embodiment, the linearized plasmid backbone DNA molecule further comprises another origin of replication capable of replicating in a selected host other than Acinetobacter, such as a for expression of the target gene.

The circularized plasmid product obtained by the method of the present invention preferably comprises at least one selection marker, wherein said selection marker is a gene that confers a trait that facilitates positive or negative selection, such as providing a host cell comprising the plasmid with a growth advantage, e.g. a gene that would confer antibiotic resistance to the host, or a visible or fluorescent selection marker, e.g. x-gal blue-white screening for β-galactosidase activity or a fluorescent protein, such as GFP. In the daily practice of recombinant DNA technology, antibiotic resistance genes are exploited as positive or negative selection elements to preferentially

enhance the amplification of the desired plasmid, and growth of host cells comprising the plasmid, over that of other plasmids. Some antibiotics are broad spectrum, i.e. inhibit growth of many different microbes, while some are more specific, only inhibiting gram negative strains.

The nucleotide sequence of the selection marker gene as described above may be comprised within the sequence of the linearized plasmid backbone DNA molecule or within the sequence of the target DNA molecule, or even in both. The selection marker gene is operably linked to a regulatory sequence that directs expression of the marker gene.

Accordingly, in one embodiment, the linearized plasmid backbone DNA molecule and/or the target DNA molecule comprises a selection marker for detection/survival of different microbes. In one embodiment, the selection marker is selective towards both gram-positive and gram-negative bacteria. In a preferred embodiment, the selection marker is selective towards gram-negative bacteria, such as gram negative bacteria selected from the list Acinetobacter, E. coli, Pseudomonas putida, Klebsiella spp., Shewanella oneidensis, and Vibrio natriegens. In a most preferred embodiment, the selection marker is selective towards Acinetobacter and E. coli. Examples of such markers selective towards Acinetobacter and E. coli are genes conferring resistance to gentamicin, spectinomycin, kanamycin.

Regulatory sequences for gene expression may be part of the linearized plasmid backbone DNA molecule and/or the DNA target molecule, and for example serve to regulate expression of the selection marker gene and/or coding sequences in the target DNA molecule. The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a Pribnow box, Shine-Dalgarno sequences, riboswitches, and the like. In particular, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably linked gene. Expression of a selected nucleic acid molecule(s) encoding the polypeptide(s) may be inducible, such as

promoters controlled by the presence or absence of a molecule, or constitutive i.e. the promoter is unregulated allowing for continual transcription of its associated gene. The promoter can be a native promoter, i.e., the promoter of the gene in its endogenous context, which provides normal regulation of expression of the gene. In some embodiments the promoter can be constitutive. Regulatory sequences may also include enhancer sequences, operators for binding transcription factors, or other upstream activator sequences as desired. The regulatory sequence may optionally include 5' leader or signal sequences. The choice and design of an appropriate expression system is within the ability and discretion of one of ordinary skill in the art.

Multiple cloning sites may also be part of the linearized plasmid backbone DNA molecule and/or the DNA target molecule. The choice and design of an appropriate multiple cloning site is within the ability and discretion of one of ordinary skill in the art.

The linearized plasmid backbone DNA molecule and/or the target DNA molecule may further comprise elements for conjugation (oriT) for transfer of DNA from one host bacterial cell to another, such as transfer of a circularized plasmid product of the invention into a bacterial cell belonging to a bacterial genus or species different from the Acinetobacter cell in which the circularized plasmid was produced. As a non-limiting example, plasmids prepared by the method of the present invention, wherein the linearized plasmid backbone DNA molecule comprises elements for conjugation may be transferred from Acinetobacter to Streptomyces by conjugation, for expression of the target DNA molecule in Streptomyces. In one embodiment, the conjugation elements originate from a gram-negative bacteria, such as Pseudomonas putida. One example of an oriT commonly used is from Pseudomonas IncP alpha plasmid (GenBank accession# BN000925. 1); this oriT is functional in most gram negative bacteria.

Table 1 provides examples of known plasmids that may be used as linearized plasmid backbone DNA molecules in the present invention for cloning and preparation of circularized plasmid in Acinetobacter. All the plasmids in Table 1 comprise origin of replication that functions in Acinetobacter.

Table 1		
Plasmid [Genbank accession#]	**Origin of replication [Genbank accession#]**	**Known host range**
pUCP24 [U07167.1]	oriV(pUC24) SEQ ID NO. 1	*Acinetobacter, E.coli, Pseudomonas*
pKLH4.05	oriV(pKLH4.05) SEQ ID NO. 2	*Acinetobacter, E.coli, Pseudomonas*
pIM1311	oriV(pWH1266)[1] SEQ ID NO. 3 [M36473.1] and pUC ori[2]	*Acinetobacter, E.coli, pseudomonas*
pME6031 [AF118811.1]	pVS1 oriV[1] SEQ ID NO. 4 and p15a ori[3] SEQ ID NO. 5	*Acinetobacter, E.coli, Pseudomonas*
pPP8-1 [AJ289784.1]	oriV(pPP8-1) SEQ ID NO. 6	*Acinetobacter, E.coli, pseudomonas*
pMEKm12	oriV(pMEKm12) SEQ ID NO. 7	*Acinetobacter, E.coli, pseudomonas*
pSEVA228 [JX560388.1]	oriV(RK2) SEQ ID NO. 8 [J01780.1].	Most gram negative bacteria
pSEVA651 [JX560350.1]	oriV(RSF1010) SEQ ID NO. 9 [M21475.1]	Most gram negative bacteria and Actinobacteria
pSEVA631 [JX560348.1]	oriV(pSEVA631) SEQ ID NO. 10	Most gram negative bacteria
pBAV1K-T5 [JF828582.1]	oriV(pBAV1K-T5) SEQ ID NO. 11	Most gram negative bacteria and gram positive bacteria

[1]ori for Acinetobacter; [2]ori for E. coli (also works in Acinetobacter but less efficiently); [3]ori for E. coli.

In a preferred embodiment, the plasmid backbone only shares very limited sequence homology with the genome of the Acinetobacter host, such as the plasmids provided in Table 1; this is to reduce the risk of homologous recombination with the host's genetic material which would then reduce the frequency of plasmid formation. Any consecutive 50 nucleic acids of the plasmid backbone sequences should therefore preferably have less than 90% homology to any region of the genome of the Acinetobacter host.

I i. *Linear DNA multimer*

In the present invention, the linear DNA multimer comprises a target DNA molecule and a linearized plasmid backbone DNA molecule, wherein at least one of these elements must be present in at least two copies. In one embodiment, the linear DNA multimer comprises at least one copy of a target DNA molecule and at least two copies of a linearized plasmid backbone DNA molecule; in another embodiment, the linear DNA multimer comprises at least one copy of a linearized plasmid backbone DNA molecule and at least two copies of a target DNA molecule. In one embodiment, the copy number of the target DNA molecule or the linearized plasmid backbone DNA molecule in the linear DNA multimer is at least 2, such as at least 3, 4, 5, 6, 7, 8, 9, 10 or more, such as between 5-50, between 10-100, between 80-200 copies. The number of copies of the target DNA molecule and linearized plasmid backbone DNA molecule in the linear DNA multimer may be the same or different.

The linear DNA multimer is preferably a double stranded, linear molecule. The DNA elements of the linear DNA multimer may be linked in any order as long as the multimer comprises at least two copies of the target DNA molecule (T) or at least two copies of the linearized plasmid backbone DNA molecule (PB).

In one embodiment, each copy of the target DNA molecule is adjacent to a copy of the linearized plasmid backbone DNA molecule, such that the structural order of the elements in the linear DNA multimer is:

(T-PB)n-T or (PB-T)n-PB or (T-PB)n-T-PB or (PB-T)n-PB-T

wherein n is at least 1, such as n is at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, such as n is between 1-35, between 5-35, between 10-35, preferably between 5-20.

One or more different target DNA molecules (Tl, T2, T3, etc.) may be combined with a linearized plasmid backbone DNA molecule (PB); facilitating cloning of multiple fragments by the present method. In one embodiment, one target DNA molecule is combined with a linearized plasmid backbone DNA molecule, forming a linear DNA multimer as described above. In another embodiment, 2, 3, 4, 5, 6, 7, 8, 9, 10 or even more target DNA molecules are combined with a linearized plasmid backbone DNA molecule, forming a linear DNA multimer. The DNA elements of the linear DNA multimer may be linked in any order as long

as the multimer comprises at least two copies of one of the target DNA molecules or the linearized plasmid backbone DNA molecule (PB).

In one embodiment, the structural order of the elements of a linear DNA multimer comprising two target DNA molecules may be

Y-(PB-Tl-T2)n-X or Z-(PB-T2-Tl)$_n$-W

wherein n is at least 1, such as n is at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, such as n is between 1-35, between 5-35, between 10-35, preferably between 5-20; Y is T1-T2, T2, or no element; X is PB-T1, PB, or no element; Z is T2-T1, T2 or no element; W is PB-T2, PB, or no element.

The order of the elements of the linear DNA multimer may be a repetitive pattern as illustrated above, but elements may in another embodiment be mixed in a non-repetitive form, such as randomly distributed. The number of copies of the different elements in the linear DNA multimer may be the same or different.

Several methods recognized by a person skilled in the art exist for preparing linear DNA multimers, including Gibson assembly and POE-PCR which are user-friendly techniques for preparing linear DNA multimers, but other methods, such as ligation, may be equally suitable.

Gibson assembly is a molecular cloning method which allows for the joining of multiple DNA molecules. The method can simultaneously combine several DNA molecules based on sequence identity. It requires that the DNA molecules contain ~20-40 base pair overlap with adjacent DNA molecules. These DNA molecules are mixed with a cocktail of enzymes (exonuclease, DNA polymerase, and DNA ligase), along with other buffer components.

Prolonged overlap extension PCR (POE-PCR) similarly allows for joining of multiple DNA molecules. In a first step, 3' and 5' overlapping DNA molecules are generated by standard PCR using primers designed specifically for this purpose; then in a second step, DNA multimers are formed in vitro by POE-PCR where multiple rounds of PCR are run without addition of primers as the DNA molecules anneal to each other and thereby provide a starting point for the elongation reaction.

Preparation of linear DNA multimers may easily be performed by robots, such as preparing several different linear DNA multimer constructs at the same time by robotic pipetting in e.g. multi-well plates

I ii. *Natural transformation in Acinetobacter*

An important aspect of the present invention concerns the step of natural transformation of Acinetobacter. Any Acinetobacter strain may be used in this step. In one embodiment, the Acinetobacter strain used in natural transformation is selected from among Acinetobacter albensis, A. apis, A. baumannii, Acinetobacter baylyi, A. beijerinckii, A. bereziniae, A. bohemicus, A. boissieri, A. bouvetii, A. brisouii, A. calcoaceticus, A. celticus, A. colistiniresistens, A. courvalinii, A. defluvii, A. disperses, A. dijkshoorniae, A. equi, A. gandensis, A. gerneri, A. guangdongensis, A. guillouiae, A. gyllenbergii, A. haemolyticus, A. harbinensis, A. indicus, A. junii, A. kookii, A. lactucae, A. larvae, A. lwoffii, A. modestus, A. nectaris, A. nosocomialis, A. parvus, A. pakistanensis, A. populi, A. proteolyticus, A. pittii, A. piscicola, A. pragensis, A. proteolyticus, A. puyangensis, A. qingfengensis, A. radioresistens, A. rudis, A. schindleri, A. seifertii, A. soli, A. tandoii, A. tjernbergiae, A. towneri, A. ursingii, A. variabilis, A. venetianus, and A. vivianii. In a preferred embodiment, Acinetobacter baylyi is used in natural transformation.

In one embodiment, a plasmid-free Acinetobacter strain is used in natural transformation, selected from among Acinetobacter sp. ADP1, Acinetobacter radioresistens DSSKY-A-001, Acinetobacter baylyi DSM 14961, Acinetobacter nosocomialis NCTC 8102, Acinetobacter pittii PHEA-2, Acinetobacter junii Izh-X15, Acinetobacter johnsonii LXL_C1, Acinetobacter guillouiae NBRC 110550, and Acinetobacter oleivorans DR1.

Amongst many of the advantageous characteristics of Acinetobacter, it is especially amendable to laboratory cloning and screening because it is nutritionally versatile, fast growing, and easily cultured.

Transformation is easily accomplished by simply adding DNA to an Acinetobacter culture; facilitating contact between the added DNA and cells of the Acinetobacter. Acinetobacter has constitutive competence. The cells are

naturally competent and thus do not need to be pre-treated to make them transformation competent. The cells may be in stationary phase or log phase during transformation. In a preferred embodiment, the cells are in log phase. The cells may be used directly from e.g. an overnight culture, such as without washing.

Divalent cation, such as Mg2+, is required for natural transformation. In one embodiment, Mg2+, Mn2+, and/or Ca2+ is added to the transformation medium to enhance natural transformation. In a preferred embodiment, transformation is carried out in a medium comprising at least 1 mM Mg2+, Mn2+, and/or Ca2+, such as at least 2, 3, 4, or 5 mM Mg2+, Mn2+, and/or Ca2+, preferably in a medium comprising between 2-10 mM Mg2+, Mn2+, and/or Ca2+. pH 6-7.5 is preferred for transformation. Standard LB medium has the right pH and most often the DNA sample itself contains a sufficient amount of Mg2+, so no additional Mg2+ will be needed.

In a further embodiment, bovine serum albumin [BSA] is added to the transformation medium to enhance the transformation, such as 0.1-2% BSA, such as 0.2-1%, preferably around 0.2% BSA.

Unpurified DNA can be used in transformation, linear DNA multimers generated by e.g. Gibson assembly of POE PCR can therefore be added directly to Acinetobacter cells suspended in the transformation medium. Incubation time (DNA with the Acinetobacter cells) is preferably at least 30 40, 50, 60 75, 90, 105, or at least 120 minutes.

Transformation may easily be performed by robots, such as performing several transformation events at the same time for preparing replicates or transform using different linear DNA multimer constructs by robotic pipetting in e.g. multi-well plates harboring Acinetobacter cells.

In one embodiment, the transformed cells of Acinetobacter are then incubated in a culture medium suitable for growth of Acinetobacter. In one embodiment, medium suitable for growth of Acinetobacter is selected from LB medium, SOC medium, and SOB medium. Acinetobacter may be plated on solid medium, and

cells carrying the plasmid of interest can be screened for by use of a selection marker encoded in the plasmid (as described previously). Plating of the cells may easily be performed by robots, which simply pipette the transformed liquid culture on top of a solid medium, with optionally shaking, and subsequent incubation. Robots equipped with a camera can further perform colony picking.

In one embodiment, the Acinetobacter culture medium prior to transformation, the transformation medium, and the culture medium post transformation are the same. In other words, linear DNA multimer is simply added to an Acinetobacter culture and cultivation is continued. In another embodiment, fresh medium may be added during transformation or post transformation cultivation, where the medium may be the same or different from the culture medium prior to transformation.

I iii. *Plasmid recovery*

The final cloned circular plasmid may be harvested from Acinetobacter by simple plasmid purification methods. A person skilled in the art would recognize that even most standard E. coli protocols work for Acinetobacter. Several plasmid recovery/purification kits may be applied for simple and easy handling. As an example, robotic protocols and kits exist for isolating plasmids from E.coli, which are also suitable for Acinetobacter.

In one embodiment, circular plasmid DNA comprising a target DNA molecule and plasmid backbone is recovered from Acinetobacter by disrupting/breaking the cell walls of Acinetobacter, such as by simply alkaline lysis.

I iii. A *method of verifying plasmids produced*

The plasmid product obtained by the method of the present invention may be verified by methods recognized by a person skilled in the art such as plasmid mapping by restriction enzyme digestion; sequencing of the plasmid; or even transforming the plasmid into a selected host (e.g. E.coli) for verification by expression of one or more genes in the plasmid.

II. Use of Acinetobacter for circularizing DNA

A second aspect of the invention concerns the use of Acinetobacter for circularizing DNA derived from exogenous linear DNA multimer molecules taken up by cells by natural transformation, wherein each said linear DNA multimer molecule comprises a target DNA molecule and a linearized plasmid backbone DNA molecule, and wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule is at least two.

III. Acinetobacter cell comprising circularized plasmid DNA

A third aspect of the invention concerns an Acinetobacter cell comprising circularized plasmid DNA, wherein said circularized plasmid DNA comprises an exogenous target DNA molecule and a linearized plasmid backbone DNA molecule. Properties of the target DNA molecule and the linearized plasmid backbone DNA molecule comprised in the circularized plasmid are described in section I of this application.

In a preferred embodiment, the Acinetobacter cell of the invention comprising circularized plasmid DNA as described above, is derived from a plasmid-free Acinetobacter strain, such as A. *baylyi.*

IV. Kit of parts for preparing circular plasmid

A fourth aspect of the invention concerns providing a kit of parts for preparing circular plasmid, wherein said kit comprises (i) Acinetobacter cells, and (ii) a plasmid backbone DNA molecule comprising an origin of replication and optionally a selection maker compatible with Acinetobacter and E. coli, as described in section I of this application.

EXAMPLES

Example 1: Gene cloning and plasmid preparation by Acinetobacter

Acinetobacter was first demonstrated to be suitable for cloning purposes using linear DNA multimers comprising the RppA (1,3,6,8-tetrahydroxynaphthalene synthase) gene cluster from Streptomyces coelicolor A3(2) and natural

transformation. The gene expression product is an easy to detect red/brown pigment.

The whole cloning process was carried out using a VANTAGE Liquid Handling robot system, including PCR, DNA multimer preparation, natural transformation of A. *baylyi,* spreading of transformants on selective agar in 6 well plates, colony PCR, and plasmid isolation.

I.a. Strain propagation

Acinetobacter baylyi strain ADP1 (BD413) was obtained from ATCC (catalog# ATCC 33305). Acinetobacter ADP1 was streaked on LB medium plate (lOg/l tryptone, 10 g/l NaCI, 5 g/l yeast extract) and incubated at 30°C for 2 days.

I.b. Preparation of DNA multimers

pUCP24 comprising lac promoter was chosen as template for preparing the linearized plasmid backbone DNA molecule; the plasmid backbone DNA molecule was amplified using primers as specified in table 2.

A 8 kb region within the RppA gene was chosen as target DNA molecule. A larger DNA fragment (approximately 9 kb), covering the target DNA molecule, was initially amplified from genomic DNA of *Streptomyces coelicolor* A3(2) using a standard PCR protocol. The desired 8 kb within the RppA gene cluster was divided into two fragments and amplified individually from the larger 9 kb template (see Figure 1). The two target DNA molecules were prepared using template and primes as specified in table 2.

Table 2. Summary of PCR fragments

Fragment name	**Fragment size (bp)**	**Template DNA**	**Primers used***
pUCP24 backbone	3941	pUCP24 linearized by BamHI digestion	pUCP24s (SEQ ID NO 12) pUCP24a (SEQ ID NO 13)
9k	9154	gDNA of *S. coelicolor* A3(2)	S0 (SEQ ID NO 14) R0 (SEQ ID NO 15)
8k half1	4098	9k fragment	S1 (SEQ ID NO 16) R2.1 (SEQ ID NO 17)
8k half2	3977	9k fragment	S2 (SEQ ID NO 18) R3 (SEQ ID NO 19)

DNA assembly of the linearized plasmid backbone DNA molecule and the two target DNA molecules was done using Gibson Assembly Cloning Kit according to the supplier protocol (New England BioLabs) or POE-PCR based on the protocol of You et al. 2011. These two methods were selected to fulfill the requirements of a high throughput technique, since they are relatively simple, easy to use, and are sequence independent. The molar ratio of linearized plasmid backbone DNA molecule to target DNA molecules was 1:5 for both methods.

Gibson Assembly: linearized plasmid backbone DNA molecule and the two target DNA molecules were mixed with the sample volume of Gibson 2X Master Mix in 50 ul reactions. The mixture was incubated at 50°C for 30 min.

POE-PCR: linearized plasmid backbone DNA molecule and the two target DNA molecules were amplified using Phusion® Hot Start Flex 2X Master Mix in 50 ul reactions. 2 ul of the target DNA molecules were mixed with 2 ul of linearized plasmid backbone DNA molecule and assembled by the same enzyme. The assembly was run at 98°C denature (15 sec), 72°C extension (5 min), for 50 cycles.

The formation of linear DNA multimers in both Gibson and POE-PCR reaction was confirmed (Figure 2). As a negative control, the linearized plasmid backbone DNA molecule alone could not form multimers (data not shown).

The assembly products (linear DNA multimers) were directly used for natural transformation of Acinetobacter.

I.c. Transformation

Acinetobacter ADP1 was grown overnight (16-24 h) in 10 ml LB medium (lOg/l tryptone, 10 g/l NaCl, 5 g/l yeast extract) in a 50 ml tube. Overnight cultures were then centrifuged at 4000 g for 5 min and cells were suspended in fresh LB medium to an OD600 of about 2.0. Five ml of this mixture was further diluted into 50 ml of fresh LB medium. This fresh mixture was used in transformation.

500 ul of the Acinetobacter culture was added into a well in a 96 deep well plate. 2 ul DNA assembly product (approx. 100 ng DNA multimer) was then added and reactions were incubated at 30°C shaking (250 rpm) overnight. 20 ul of the culture was diluted 1, 10, and 100 times and spread on LB agar plates containing gentamycin and incubated at 30°C for 2 days. 5 colonies were picked from the plates, and the correct insert was confirmed by colony PCR (Figure 3).

I.d. Plasmid extraction

Plasmid extraction/purification was performed using NucleoSpin®Plasmid (Macheey-Nagel), following the protocol provided by the manufacture, where plasmid is liberated from the host cell by SDS/alkaline lysis buffer.

I.e Detecting RppA plasmids by RppA expression in E. coli.

The purified circular plasmids were transformed into E. coli BL21(DE3) chemical competent cells by a standard heat shock method. Cells were grown in liquid medium and pigment production was visually observed by the culture medium turning red/brownish, thereby indicating expression of the RppA fragment from the circular plasmid.

This study was the first to use Acinetobacter as a platform for cloning DNA into circular plasmid.

Example *2:* Plasmids constructed by Acinetobacter natural transformation

The novel method of the present invention was used for cloning gene clusters from *Streptomyces, Pseudomonas,* and *Photorhabdus* genomes. The selected gene cluster complied with two criteria: I) their size should accommodate standard PCR amplification and II) the gene clusters encodes enzymatic pathways for small molecular biosynthesis. These gene clusters had a size range from 0.6 - 16 kb, and GC range from 24% to 73%. They were cloned as a target DNA molecule or divided into two target DNA molecules.

Table 3 provides a summary of the different circular plasmids successfully constructed by Acinetobacter natural transformation. The linear DNA multimers

comprising linearized plasmid backbone DNA molecules and target DNA molecules were prepared either by Gibson assembly or POE-PCR as described in Example 1. The linear DNA multimer product was used directly (no purification) in transformation by mixing with the Acinetobacter ADP1 culture and incubating on suitable selective medium. Circular plasmids were isolated by a standard procedure; and then introduced into E. coli BL21 (DE3) to allow detection of gene cluster expression.

Studies on integration of chromosomal or plasmid DNA within Acinetobacter genomic DNA (not circular plasmid formation) used chromosomal DNA or plasmid DNA having an around 50% GC-content as transformant DNA material [Genoscope 2008; Nielsen et al 1997; Gebhard et al 1998], however the present invention demonstrates the ability of A. baylyi ADP1 to handle much higher GC content in plasmid formation. Although A. baylyi ADP1 has a low GC content (around 40%) in its own genome, it was found capable of converting high GC content DNA (up to 72% tested) into plasmids, such as the rppA gene cluster cloned in Example 1.

Table 3. Summary of different plasmids constructed by Acinetobacter natural transformation

Circular plasmid product	Linearized plasmid backbone DNA molecule*				Target DNA molecule(s)*				
	Name ID	size (bp)	template	primers	Name ID	size (pb)	template	primers	Identify/origin of target DNA molecule
pUCP-1.5k	pUCP24 backbone	3941	pUCP24[1] linearized by BamHI digestion	pUCP24s (SEQ ID NO 12) pUCP24a (SEQ ID NO 13)	1.5k	1537	gDNA of *S.coelicolor A3(2)*	S1 (SEQ ID NO 16) R1 (SEQ ID NO 20)	RppA gene cluster (partial) from *Streptomyces*
pUCP-4k	pUCP24 backbone	3941	pUCP24[1] linearized by BamHI digestion	pUCP24s (SEQ ID NO 12) pUCP24a (SEQ ID NO 13)	4k	4098	gDNA of *S.coelicolor A3(2)*	S1 (SEQ ID NO 16) R2 (SEQ ID NO 21)	RppA gene cluster from *Streptomyces*
pUCP-8k	pUCP24 backbone	3941	pUCP24[1] linearized by BamHI digestion	pUCP24s (SEQ ID NO 12) pUCP24a (SEQ ID NO 13)	8k half1	4098	gDNA of *S.coelicolor A3(2)*	S1 (SEQ ID NO 16) R2.1 (SEQ ID NO 17)	RppA gene cluster (extended) from *Streptomyces*
					8k half2	3977	gDNA of *S.coelicolor A3(2)*	S2 (SEQ ID NO 18) R3 (SEQ ID NO 19)	RppA gene cluster (extended) from *Streptomyces*
pUCP-Pspls	pUCP24 backbone 1	3879	pUCP24[1] linearized by BamHI digestion	Xj332 (SEQ ID NO 22) Xj333 (SEQ ID NO 23)	Pspls	4722	gDNA of *P.syringae* DSM 50252	Xj330 (SEQ ID NO 24) Xj331 (SEQ ID NO 25)	Potential polylysine biosynthetic gene cluster from *Pseudomonas*
pXJ100-RiPP1.1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP1.1	2685	gDNA of *Streptomyces* Nai2	Xj280 (SEQ ID NO 28) Xj281 (SEQ ID NO 29)	RppA gene cluster (extended) from *Streptomyces*

pXJ100-RiPP1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP1	4682	gDNA of *Streptomyces* Nai2	xj282 (SEQ ID NO 30) Xj283 (SEQ ID NO 31)	RiPP gene cluster from *Streptomyces*
pXJ1000-RiPP2	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RIPP 2	6744	gDNA of *Streptomyces* Nai2	Xj214 (SEQ ID NO 32) Xj215 (SEQ ID NO 33)	RIPP 2 class III lantipeptide, RiPP gene cluster from *Streptomyces*
pXJ100-RiPP3	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3	6425	gDNA of *Streptomyces* Nai2	Xj216 (SEQ ID NO 34) Xj217 (SEQ ID NO 35)	RiPP gene cluster from *Streptomyces*
pXJ100-RiPP5	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP5	8927	gDNA of *Streptomyces sp.* NRRL F-5053	Xj220 (SEQ ID NO 36) Xj221 (SEQ ID NO 37)	nai33 cluster 3, RiPP gene cluster from *Streptomyces*
pXJ100-RiPP6	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP6	4434	gDNA of *Streptomyces sp.* NRRL F-5053	Xj222 (SEQ ID NO 38) Xj223 (SEQ ID NO 39)	nai33 cluster 37, RiPP gene cluster from *Streptomyces*
pXJ100-RiPP7	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP7	4434	gDNA of *Streptomyces sp.* NRRL F-5053	Xj224 (SEQ ID NO 40) Xj225 (SEQ ID NO 41)	nai33 cluster 38, RiPP gene cluster from *Streptomyces*
pXJ100-RiPP8	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP8	8836	gDNA of *Streptomyces rimosus* ATCC	Xj226 (SEQ ID NO 42) Xj227 (SEQ ID NO 43)	rimosus cluster 17, Class I lantipeptide, RiPP gene cluster from *Streptomyces*

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pXJ100-RiPP7.1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP7.1	3996	gDNA of *Streptomyces sp.* NRRL F-5053	Xj284 (SEQ ID NO 44) Xj285 (SEQ ID NO 45)	RiPP gene cluster from *Streptomyces*
pXJ100-RiPP7in1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	ladder of RiPP1+ lasso peptide of RiPP7		Chemically synthesized (SEQ ID NO 46)		RiPP gene cluster from *Streptomyces,* refactored
					CB of RiPP1	2552	gDNA of *Streptomyces* Nai2	Xj286 (SEQ ID NO 47) Xj287 (SEQ ID NO 48)	RiPP gene cluster from *Streptomyces,*
pXJ100-RiPP1in7	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	ladder of RiPP7+lasso peptide of RiPP1		Chemically synthesized (SEQ ID NO 49)		RiPP gene cluster from *Streptomyces,* refactored
					CBM of RiPP7	3783	gDNA of *Streptomyces sp.* NRRL F-5053	Xj288 (SEQ ID NO 50) Xj289 (SEQ ID NO 51)	RiPP gene cluster from *Streptomyces,*
pXJ100-CaulosegninIin1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	ladder of RiPP1+ lasso peptide of		Chemically synthesized (SEQ ID NO 52)		RiPP gene cluster from *Streptomyces,* refactored

					Cauloseg nin I				
					CB of RiPP1	2552	gDNA of *Streptomyc es* Nai2	Xj286 (SEQ ID NO 47) Xj287 (SEQ ID NO 48)	RiPP gene cluster from *Streptomyces,*
pXJ100-RiPP3.1	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3 3inI	229	gDNA of *Streptomyc es* Nai2	Xj292 (SEQ ID NO 53) Xj293 (SEQ ID NO 54)	RiPP gene cluster from *Streptomyces,*
					RiPP3 A	2694	gDNA of *Streptomyc es* Nai2	Xj290 (SEQ ID NO 55) Xj291 (SEQ ID NO 56)	RiPP gene cluster from *Streptomyces,*
pXJ100-RiPP3.2	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3 3inII	223	gDNA of *Streptomyc es* Nai2	Xj294 (SEQ ID NO 57) Xj295 (SEQ ID NO 58)	RiPP gene cluster from *Streptomyces,*
					RiPP3 A	2694	gDNA of *Streptomyc es* Nai2	Xj290 (SEQ ID NO 55) Xj291 (SEQ ID NO 56)	RiPP gene cluster from *Streptomyces,*
pXJ100-RiPP3.3	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3 3inIII	239	gDNA of *Streptomyc es* Nai2	Xj296 (SEQ ID NO 59) Xj297 (SEQ ID NO 60)	RiPP gene cluster from *Streptomyces,*
					RiPP3 A	2694	gDNA of *Streptomyc es* Nai2	Xj290 (SEQ ID NO 55) Xj291 (SEQ ID NO 56)	RiPP gene cluster from *Streptomyces,*

pXJ100-RiPP3.4	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3 3inV	235	gDNA of *Streptomyces* Nai2	Xj298 (SEQ ID NO 61) Xj299 (SEQ ID NO 62)	RiPP gene cluster from *Streptomyces,*
					RiPP3 A	2694	gDNA of *Streptomyces* Nai2	Xj290 (SEQ ID NO 55) Xj291 (SEQ ID NO 56)	RiPP gene cluster from *Streptomyces,*
pXJ100-RiPP3.5	pXJ100 backbone	3519	pXJ100[2] linearized by EcoRI digestion	Xj210 (SEQ ID NO 26) Xj211 (SEQ ID NO 27)	RiPP3 3inI-V	978	gDNA of *Streptomyces* Nai2	Xj300 (SEQ ID NO 63) Xj301 (SEQ ID NO 64)	RiPP gene cluster from *Streptomyces,*
					RiPP3 A	2694	gDNA of *Streptomyces* Nai2	Xj290 (SEQ ID NO 55) Xj291 (SEQ ID NO 56)	RiPP gene cluster from *Streptomyces,*
pXJ100-NrpsNu1	pXJ100 backbone 1	3549	pXJ100[2] linearized by EcoRI digestion	xj306 (SEQ ID NO 65) xj307 (SEQ ID NO 66)	NrpsNu1 half1	10710	gDNA of *Photorhabdus asymbiotica* ATCC43949	xj302 (SEQ ID NO 67) xj303 (SEQ ID NO 68)	Potential nucleoside antibiotic biosynthetic gene cluster from *Photorhabdus asymbiotica*
					NrpsNu1 half2	5437	gDNA of *Photorhabdus asymbiotica* ATCC43949	xj304 (SEQ ID NO 69) xj305 (SEQ ID NO 70)	Potential nucleoside antibiotic biosynthetic gene cluster from *Photorhabdus asymbiotica*

pXJ100-nucleoside1	pXJ100 backbone 1	3549	pXJ100[2] linearized by EcoRI digestion	xj306 (SEQ ID NO 65) xj307 (SEQ ID NO 66)	nucleoside1	18797	gDNA of *Xenorhabdus szentirmaii* DSM 16338	xj308 (SEQ ID NO 71) xj309 (SEQ ID NO 72)	Potential nucleoside antibiotic biosynthetic gene cluster from *Xenorhabdus szentirmaii*
pXJDP-cmr-plac-gfp (pXJ160)	pXJDP backbone	7514	pXJDP[3] linearized by BamHI and NcoI digestion	fbxj100 (SEQ ID NO 73) rbxj100.1 (SEQ ID NO 74)	Cmr-plac-gfp	1700	pXJ157[4]	Xj466 (SEQ ID NO 75) Xj467 (SEQ ID NO 76)	green fluorescent protein gene as a reporter gene

*The linearized plasmid backbone DNA molecule and target DNA molecules were prepared by PCR from DNA template using primers as listed in the table, or chemically synthesized as indicated in the table.

[1] pUCP24 reference: West et al 1994; [2'3'4] pXJOOO, pXJDP, pXJ157: in-house plasmids, constructed with a different promoter system, but having the same replication origins (oriV and pUC ori) and selection marker (gentamycin resistance) as pUCP24.

This study demonstrated that Acinetobacter can be used for high efficiency cloning of both single and multiple genes and gene clusters; facilitating the option of cluster refactoring. The Gibson assembly and POE-PCR were found to be user and automation friendly techniques, yielding reactions products (linear DNA multimers) suitable for direct Acinetobacter natural transformation without the need for purification. In conclusion, A. baylyi ADP1 is a powerful platform for high through-put cloning.

Example 3: Comparing cloning efficiency of Acinetobacter ADP1, E.coli and Bacillus subtilis using hybrid backbone

Both Acinetobacter ADP1 and Bacillus subtilis are naturally competent. To directly compare their efficiency for gene cloning, the two bacteria were transformed with the same linear DNA multimer comprising linearized plasmid backbone DNA molecule and target DNA molecule. For this purpose, a linearized plasmid backbone DNA molecule comprising two origin of replications that can replicate in each of them (as well as in E. coli) was prepared from pXJlOO (plasmid that can replicate in Acinetobacter ADP1 and E.coli) and pDP66K-Pveg-sfGFP (standard plasmid vector for Bacillus). This "hybrid" linearized plasmid backbone DNA molecule (named as pXJDP) was initially prepared as a circular plasmid by standard cloning procedures in E. coli by simple amplification of the two plasmids (using primers as specified in table 4), Dpnl restriction digestion, Gibson assembly, transformation into E.coli, and selection on gentamycin- and kanamycin- supplemented LB plates. The pXJDP plasmid DNA was purified and then restriction digested by Hindlll, BamHI, and Ncol, respectively, confirming it was correctly constructed.

Table 4

Fragment	**template**	**Primer**
pDP66K fragment (3814bp)	pDP66K-Pveg-sfGFP	Xj491 (SEQ ID NO 77) Xj492 (SEQ ID NO 78)
pXJ100 fragment (3686bp)	pXJ100	Xj493 (SEQ ID NO 79) Xj494 (SEQ ID NO 80)

To compare the cloning efficiency of Acinetobacter ADP1, Bacillus subtilis and E. coli, the hybrid pXJDP was used for cloning of a 1700bp target DNA molecule: cmr-plac-gfp, comprising chloramphenicol resistance and GFP genes with pLac promoter. The linearized plasmid backbone DNA molecule and target DNA molecule were each amplified as specified in table 5 and assembled into a linear DNA multimer by POE-PCR, as described previously.

Table 5

Fragment	**template**	**Primer**
cmr-plac-gfp (1700bp)	pXJ157	Xj466 (SEQ ID NO 75) Xj467 (SEQ ID NO 76)
pXJDP (7.5kb)	linearized pXJDP	fbxj100 (SEQ ID NO 73) rbxj100.1 (SEQ ID NO 74)

For Acinetobacter, natural transformation was carried out as follows: Acinetobacter ADP1 was grown overnight (16-24 h) in 10 ml LB medium in 50 ml tube. Overnight cultures were then centrifuged at 4000 g for 5 min and cells were suspended in fresh LB medium to an OD600 about 2.0. 5ml of this mixture was diluted into 50 ml of fresh LB medium. 500 ul of the mixture was added into a well in 96 deep well plate. Transforming DNA (250 ng DNA multimer) was then added and reactions were incubated at 30。C shaking (250 rpm) overnight. One tenth of the transformation culture was spread on gentamycin-supplemented LB agar plates.

For Bacillus subtilis, natural transformation was carried out exactly as described in Zhang et al 2014 using super-competent xylose inducible B. subtilis SCK6 cells. 250 ng DNA multimer was used in transformation and one tenth of the transformation culture was spread on kanamycin LB agar plates

For E. coli, electrotransformation was carried out as follows: E.coli TopIO competence cell was purchased from Thermofisher (Catalog number: C404050). E.coli TopIO was transformed with 250 ng assembly product by electroporation using 1 mm cuvette, at 1.8 KV on an Eppendorf Eporator®. One tenth of the transformation culture was spread on gentamycin-supplemented LB agar plates.

Results of the transformations can be seen in Figure 4. Colonies were randomly picked from each transformation and checked by colony PCR. The results are summarized in table 6.

Table 6

Cloning method	Colony number per 1ug DNA	Cloning positive ratio*
E.coli Top10 Electrocompetent cells	1440 ± 98	58%
Acinetobacter natural transformation	3570 ± 2270	100%
Bacillus subtilis SCK6 natural transformation	130 ± 80	100%

^Confirmed by colony PCR.

In conclusion, it was found that Acinetobacter ADP1 showed 27 times higher cloning efficiency than B. subtilis SCK6. Zhang et al 2017 report transformation efficiencies of l-3xl0 ^{4} per ug DNA multimer for Bacillus; however, it is noted that the plasmid product prepared by Zhang et al 2017 is composed of a target DNA molecule (930 bp) and plasmid backbone (pET20b, approx. 3700 bp) yielding a much smaller plasmid than the pXJDP-cmr-plac-gfp plasmid (approx. 9200bp) prepared in the present experiment. Acinetobacter is thereby also shown to be superior for constructing large plasmids.

Both Acinetobacter and B. subtilis showed very high positive colony ratio (no false positive colonies were identified), while E. coli showed a positive colony ratio of only 58%.

Example 4: Acinetobacter natural transformation results in fewer false positive clones than E. coli transformation

E.coli electroporation and *Acinetobacter* natural transformation was prepared for cloning the same DNA targets:

4.1 *pUCPSk*

The Gibson assembly reaction of pUCP-8k (prepared as described in example 1) was used to transform *E. coli* DH5a electrocompetent cells [Dower et al 1988] and *Acinetobacter* natural competent cells. As summarized in table 3, pUCP-8k is composed of pUCP24 backbone and inserts 8k halfl and 8k half2 of

the RppA gene cluster from *Streptomyces.* Results of the transformation comparison reported in table 7, show that although a significantly larger number of colonies were obtained on the selective plates using E. coli electrocompetent cells when compared to using Acinetobacter, none of the tested E. coli transformants had the correct plasmid as tested by colony PCR. While, relatively fewer Acinetobacter transformants were found on the selective plate; importantly, it was confirmed that 8 out of 10 of the tested Acinetobacter transformants had the correct plasmid.

Table 7

Cloning method	**Colony number per 1ug DNA**	**Cloning positive ratio***
E.coli DH5α electrocompetent cells	2630±268	0/10 are correct
Acinetobacter natural competent cells	150±32	8/10 are correct

^Confirmed by colony PCR.

4.2 *pXJDP-cmr-plac-gfp*

The POE-PCR assembly reaction of pXJDP-cmr-plac-gfp (prepared as described in example 3) was used to transform *E.coli* ToplO electrocompetent cells and Acinetobacter natural competent cells. Results of the transformation comparison were reported in table 6 in example 3. Acinetobacter transformation resulted in approximately twice as many colonies per microgram DNA compared to E. coli electrocompetent cells. Further, Acinetobacter also performed better in terms of having the greatest number of correct transformants as all tested Acinetobacer transfomants had the correct plasmid compared to only 58% of the E. coli transformants being correct.

EXAMPLE 5: Acinetobacter transformation frequency is improved using linear DNA multimers vs circular DNA.

The transformation capabilities of A. baylyi ADP1 WT and ADPlHsdR: :cat were investigated by comparing transforming with circular or linear DNA multimer from pucp24 (small plasmid, 4036 bp) and pXJ100-NNl (large plasmid, 19663 bp). The circular DNA was prepared from E.coli, while the linear DNA mutimer was prepared by phi29 DNA polymerase amplification.

Results: A significant difference in transformation frequency was observed when transforming the cells using circular DNA vs linear multimer DNA: linear DNA multimers are much better than circular DNA as substrate for A. baylyi transformation (Table 8). In all cases, the transformation frequencies for ADPlHsdR: :cat strain were no greater than the wild type strain.

Table 8. Transformation frequency of *A. baylyi* ADP1 WT and ADP1 HsdR::cat with linear multimer and circular plasmid DNA: Colony number per ug DNA

	pucp24		pXJA1(pXJ100-NN1)	
	circular DNA	linear multimer	circular DNA	linear multimer
Acinetobacter ADP1	$1.80 \pm 0.78 \times 10^4$	$1.14 \pm 0.06 \times 10^7$	0	$4.93 \pm 2.59 \times 10^3$
Acinetobacter ADP1 HsdR::cat	$1.10 \pm 0.08 \times 10^4$	$0.89 \pm 0.11 \times 10^7$	0	$3.60 \pm 0.99 \times 10^3$

In the present application it has been shown that linear DNA multimers prepared by POE-PCR or Gibson reaction can be used directly for the natural transformation of Acinetobacter, and that linear DNA multimers have a much higher efficiency for Acinetobacter natural transformation than circular plasmid.

Without being bound by theory, during Acinetobacter natural transformation, DNA is taken up by the cell by means of digesting one of the DNA strands while transporting the other strand across the cells membrane into the cytoplasm. For a circular plasmid [cP] we speculate that DNA is therefore first processed into a "linear form" [IP], or at least nicked, for its transport into the cells as a single strand [ssIP], and then within the cell processed back to the circular plasmid form [cP]. On the contrary, the DNA multimer [M] used in the present invention is already in linear form and can directly be transported into the cells as a single strand multimer [ssM], wherein it is processed into a circular plasmid form. The single stranded linearized plasmid [ssIP] and the single stranded multimer [ssM] are circularized to the same final product, but the different means of circularization are unknown.

Previous studies found (data not shown) that for efficient transformation, the transformed DNA should indeed be in the form of a linear multimer rather than a linear single copy construct, as natural transformation using a linear single copy DNA construct is much less efficient than transformation using linear multimer DNA molecules.

No studies report the exact mechanism of circularization within the cell of single stranded linearized plasmid [ssIP] (i.e. single copy construct) or single stranded multimer [ssM]. But from the present findings in this application, it is evident that looking at the end result, the transformation using linear DNA multimer has a much higher efficiency in generating circularized plasmid.

REFERENCES

Dower, W. J., et al. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Research 16(13): 6127-6145 (1988).

Durot M, et al. Iterative reconstruction of a global metabolic model of Acinetobacter baylyi ADP1 using high-throughput growth phenotype and gene essentiality data. BMC Syst Biol. 2008;2(1):85. doi: 10. 1186/1752-0509-2-85.

Genoscope CN de S. Acinetobacter baylyi. A versatile bacterium which is highly competent for natural transformation. http://www.genoscope.cns.fr/spip/Acinetobacter-baylyi-highly.html. Published 2008.

Gebhard F, Smalla K. Transformation of Acinetobacter sp. strain BD413 by transgenic sugar beet DNA. Appl Environ Microbiol. 1998;64(4): 1550-1554.

Lucidi M et al 2018. New Shuttle Vectors for Gene Cloning and Expression in Multidrug-Resistant Acinetobacter Species. ASM Antimicrobial Agents and Chemotherapy. April 2018 Volume 62 Issue 4.

Melnikov, A. et al. Random mutagenesis by recombinational capture of PCR products in Bacillus subtilis and Acinetobacter calcoaceticus. Nucleic Acids Research 27(4): 1056-1062 (1999).

Metzgar D, et al. Acinetobacter sp. ADP1 : an ideal model organism for genetic analysis and genome engineering. Nucleic Acids Res. 2004;32(19): 5780-5790. doi: 10.1093/nar/gkh881.

Nielsen KM, et al. Natural transformation and availability of transforming DNA to Acinetobacter calcoaceticus in soil microcosms. Appl Environ Microbiol. **1997;63(5):** 1945-1952

You et al. Simple cloning via direct transformation of PCR product (DNA multimer) to Escherichia coli and Bacillus subtilis. Applied and Environmental Micobiology 2012, 78:5, p. 1593-1595 (published ahead of print Dec **2011)**

West SEH, et al. Construction of improved Escherichia-Pseudomonas shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in Pseudomonas aeruginosa. Gene. 1994;148(l):81-86. doi: 10.1016/0378-1119(94)90237-2

Zhang et al. Transformation of Bacillus subtilis. May **2014.** Methods in molecular biology (Clifton, N.J.) 1151 :95-101. DOI: 10.1007/978-1-4939-0554-6_7

CLAIMS

1. A method for preparing circular plasmid DNA, said method comprising the steps of:
   a) providing a linear DNA multimer comprising (I) a target DNA molecule and (II) a linearized plasmid backbone DNA molecule, wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least two,
   b) contacting Acinetobacter cells with said linear DNA multimer,
   c) recovering circularized plasmid DNA from naturally transformed Acinetobacter cells obtained from step (b), wherein said circularized plasmid DNA comprises said target DNA molecule and said linearized plasmid backbone DNA molecule.

2. The method for preparing circular plasmid DNA according to claim 1, comprising an additional step (b') of (i) plating cells of Acinetobacter obtained from step (b) on solid medium, and (ii) identifying Acinetobacter cell colonies harboring circularized plasmid, wherein said circularized plasmid DNA comprises said target DNA molecule and said linearized plasmid backbone DNA molecule; and wherein the Acinetobacter cells in step (c) are the cells identified in step (b')(ii).

3. The method for preparing circular plasmid DNA according to claim 1 or 2, wherein said Acinetobacter is Acinetobacter baylyi.

4. The method for preparing circular plasmid DNA according to any one of claims 1-3 , wherein at least steps (b) and (c) are automated.

5. The method for preparing circular plasmid DNA according to any one of claims 1-4, wherein said linearized plasmid backbone DNA molecule comprises an origin of replication compatible with a gram negative bacterium, such as E. coli.

6. The method for preparing circular plasmid DNA according to claim 5, wherein said linearized plasmid backbone DNA molecule further

comprises a selection marker compatible with a gram negative bacterium, such as E. coli.

7. The method for preparing circular plasmid DNA according to any one of claims 1-6, wherein the copy number of said target DNA molecule or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least 5.

8. The method for preparing circular plasmid DNA according to any one of claims 1-7, wherein each copy of said target DNA molecule is adjacent to a copy of said linearized plasmid backbone DNA molecule in said linear DNA multimer.

9. The method for preparing circular plasmid DNA according to any one of claims 1-8, wherein transformation in step (b) is performed in a medium comprising Bovine Serum Albumin and/or divalent cation selected from among Mg^{2+}, Mn^{2+} and Ca^{2+}.

10. Use of Acinetobacter cells for preparing circular plasmid DNA from an extracellular linear DNA multimer, wherein said DNA multimer comprises (I) a target DNA molecule and (II) a linearized plasmid backbone DNA molecule, and wherein the copy number of said target DNA molecule and/or said linearized plasmid backbone DNA molecule in said linear DNA multimer is at least two.

11. A cloning kit for preparing circular plasmid DNA, said kit comprising (i) Acinetobacter cells, and (ii) a plasmid backbone DNA molecule comprising an origin of replication and optionally a selection maker compatible with Acinetobacter and E. coli.

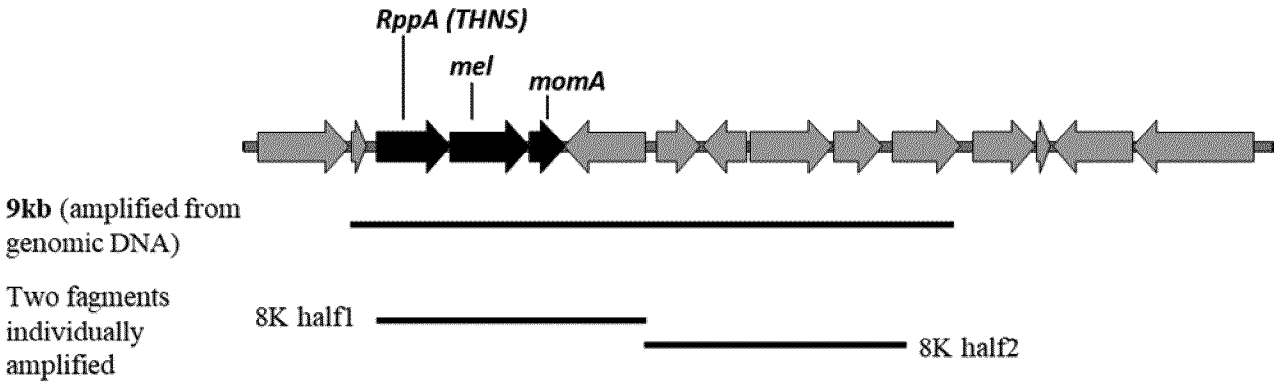

FIGURE 1

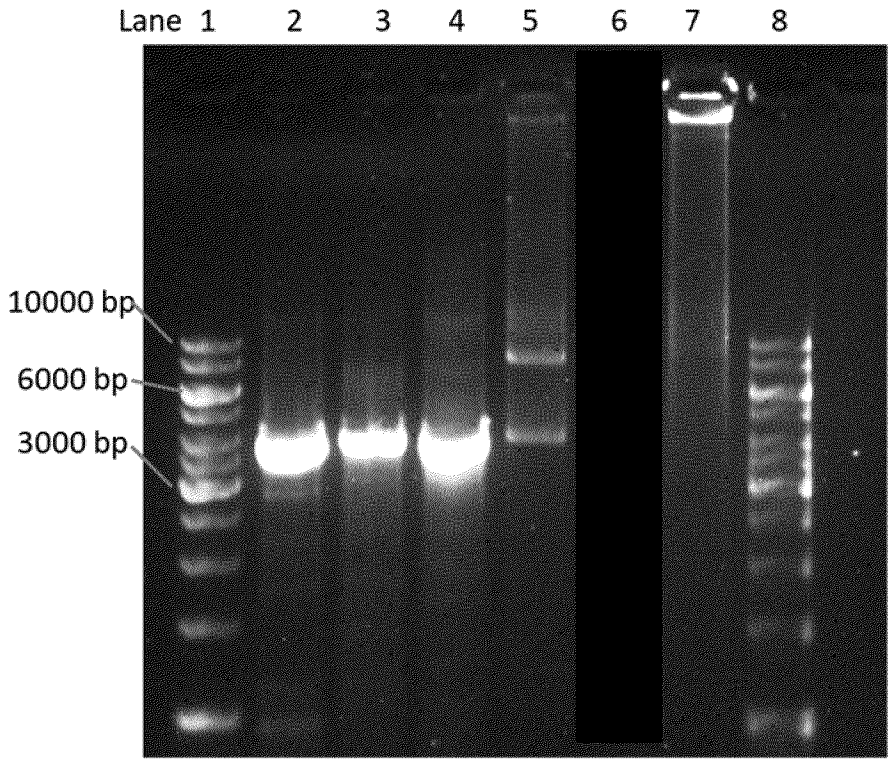

FIGURE 2

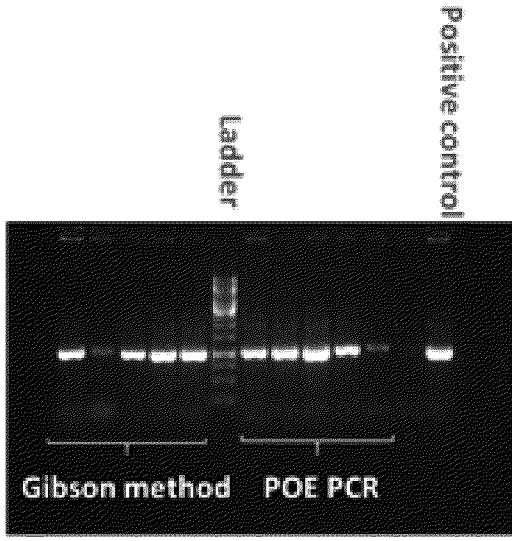

FIGURE 3

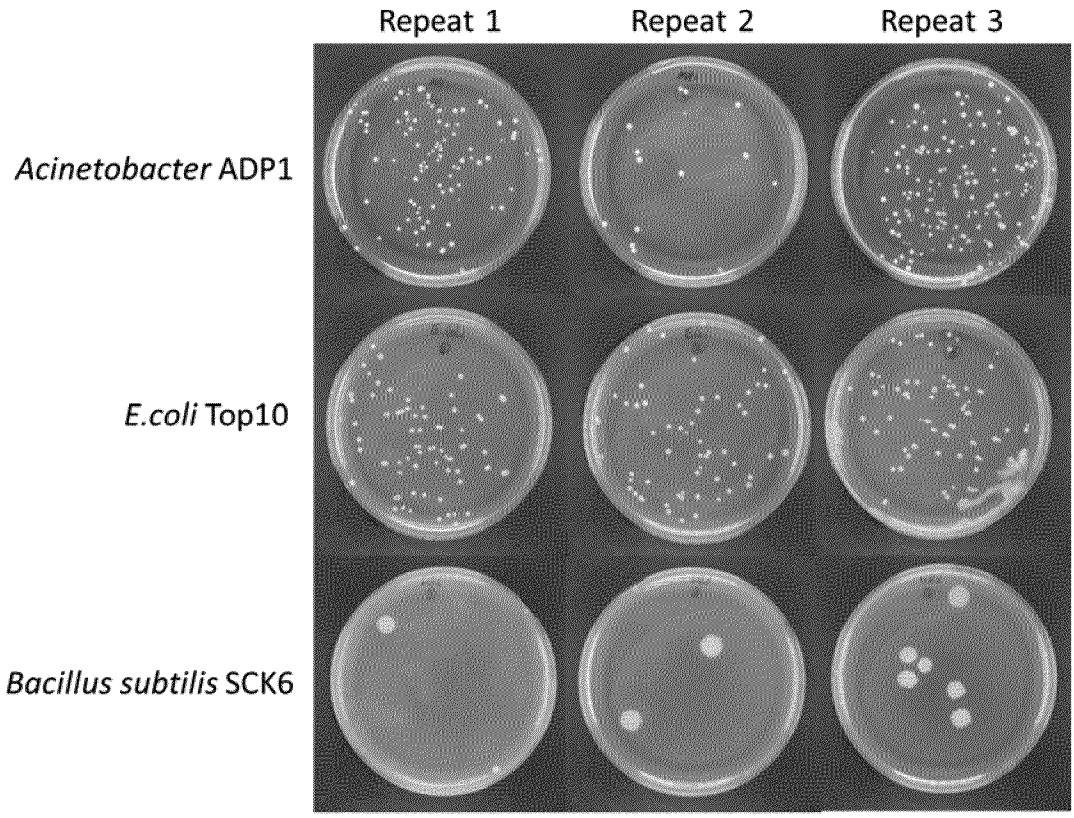

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/ EP2020/05858 1

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N 15/63 C12N 15/70 C12N 15/74
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2017/025447 A1 (CUREVAC AG [DE]) 16 February 2017 (2017-02-16) see claims 1, 22 and 26, 36-37 -----	1-11
Y	WO 2006/023546 A2 (NATURE TECHNOLOGY CORP [US]) 2 March 2006 (2006-03-02) see claims 1 and 9 and pages 3-7 -----	1-11
Y	CUNNINGHAM DREW S ET AL: "Factors affecting plasmid production in Escherichia coli from a resource allocation standpoint", MICROBIAL CELL FACTORIES,, vol. 8, no. 1, 22 May 2009 (2009-05-22), page 27, XP021058467, ISSN: 1475-2859, DOI: 10.1186/1475-2859-8-27 see abstract and pages 5-10 -----	1-11
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2020	24/06/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vix, Olivier

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAM NARAYAN TRIVEDI ET AL: "High-Level Production of Plasmid DNA by Escherichia coli DH5alpha Omega sacB by Introducing inc Mutations", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 80, no. 23, 1 December 2014 (2014-12-01), pages 7154-7160, XP055616844, US ISSN: 0099-2240, DOI: 10.1128/AEM.02445-14 see introduction and pages 7155-7158	1-11
X	MASSIMILIANO LUCIDI ET AL: "New Shuttle Vectors for Gene Cloning and Expression in Multidrug-Resistant Acinetobacter Species", ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 62, no. 4, 16 January 2018 (2018-01-16), XP055622697, US ISSN: 0066-4804, DOI: 10.1128/AAC.02480-17	1-3,5,6, 8,10
Y	see abstract, pages 2-8 and tables 1,2,3	1-11
Y	Karen Billeci ET AL: "Implementation of an Automated High-Throughput Plasmid DNA Production Pipeline", Journal of Laboratory Automation Society for Laboratory Automation and Screening, 1 January 2016 (2016-01-01), pages 765-778, XP055623030, Retrieved from the Internet: URL:https://journals.sagepub.com/doi/pdf/10.1177/2211068216630547 [retrieved on 2019-09-17] the whole document	1-11
Y	GHEORGHE M BORJA ET AL: "Engineering Escherichia coli to increase plasmid DNA production in high cell-density cultivations in batch mode", MICROBIAL CELL FACTORIES,, vol. 11, no. 1, 19 September 2012 (2012-09-19), page 132, XP021121347, ISSN: 1475-2859, DOI: 10.1186/1475-2859-11-132 see abstract and pages 4-8	1-11

-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INÊS CHEN ET AL: "DNA uptake during bacterial transformation", NATURE REVIEWS. MICROBIOLOGY, vol. 2, no. 3, 1 March 2004 (2004-03-01), pages 241-249, XP055617293, GB ISSN: 1740-1526, DOI: 10.1038/nrmicro844 see pages 241-244	1-11
Y	C. YOU ET AL: "Simple Cloning via Direct Transformation of PCR Product (DNA Multimer) to Escherichia coli and Bacillus subtilis", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 78, no. 5, 1 March 2012 (2012-03-01), pages 1593-1595, XP055378419, US ISSN: 0099-2240, DOI: 10.1128/AEM.07105-11 cited in the application the whole document	1-11
Y	MELNIKOV A ET AL: "Random mutagenesis by recombinational capture of PCR products in Bacillus subtilis and Acinetobacter calcoaceticus", NUCLEIC ACIDS RESEARCH, INFORMATION RETRIEVAL LTD, vol. 27, no. 4, 15 February 1999 (1999-02-15), pages 1056-1062, XP002355311, ISSN: 0305-1048, DOI: 10.1093/NAR/27.4.1056 cited in the application the whole document	1-11
A	K M Nielsen ET AL: "Natural transformation and availability of transforming DNA to Acinetobacter calcoaceticus in soil microcosms", Applied and Environmental Microbiology, 1 May 1997 (1997-05-01), pages 1945-1952, XP055616687, United States Retrieved from the Internet: URL:https://aem.asm.org/content/aem/63/5/1945.full.pdf cited in the application the whole document	1-11
A	EP 2 848 691 A1 (WESTFÄLISCHE WILHELMS UNIVERSITÄT MÜNSTER [DE]) 18 March 2015 (2015-03-18) the whole document	1-11

-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MATTI KANNISTO ET AL: "ABSTRACT", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 80, no. 22, 5 September 2014 (2014-09-05), pages 7021-7027, XP055617567, US ISSN: 0099-2240, DOI: 10.1128/AEM.01837-14 the whole document -----	1-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/058581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017025447 A1	16-02-2017	CN 108138187 A	08-06-2018
		EP 3334828 A1	20-06-2018
		US 2019225971 A1	25-07-2019
		WO 2017025447 A1	16-02-2017
WO 2006023546 A2	02-03-2006	AU 2005277510 A1	02-03-2006
		BR PI0515210 A	08-07-2008
		CA 2576939 A1	02-03-2006
		CN 101076597 A	21-11-2007
		EA 200700416 A1	26-10-2007
		EP 1781800 A2	09-05-2007
		IL 181410 A	31-05-2011
		JP 5112866 B2	09-01-2013
		JP 2008510460 A	10-04-2008
		KR 20070053765 A	25-05-2007
		NZ 553945 A	26-03-2010
		US 2007254342 A1	01-11-2007
		US 2011281304 A1	17-11-2011
		WO 2006023546 A2	02-03-2006
EP 2848691 A1	18-03-2015	EP 2848691 A1	18-03-2015
		US 2015072898 A1	12-03-2015