Title: GENOME SEQUENCE BASED TARGETED CLONING OF DNA FRAGMENTS

Abstract: The present invention belongs to the field of molecular biology, microbiology and microbial genetics and specifies the plasmid rescue method for targeted cloning of DNA fragments from a donor organism into a host cell, comprising inter alia the steps of transferring a Bacterial Artificial Chromosome (BAC)-vector that comprises a site-specific recombination system containing an integrase and an attP site into a donor organism, wherein said donor organism does not contain a functional attB site. Further, the present invention refers to a plasmid rescue method for targeted cloning of DNA fragments from a donor organism into a host cell, comprising inter alia the steps of transferring a BAC-vector that comprises a site-specific recombination system containing an attP site however no functional integrase, and to a vector comprising inter alia a site-specific recombination system containing an integrase and an attP site and a DNA sequence suitable for homologous recombination. The present invention further refers to the use of said vectors for cloning and/or transferring of a DNA fragment. Additionally, the present invention refers to suitable donor organisms comprising the BAC vectors according to the present invention, to an expression system comprising a host cell and a BAC vector, as well as to the use of said expression system for cloning and/or transfer of the DNA fragment, and/or for expression of said DNA fragment.
Field of the invention

The present invention belongs to the field of molecular biology, microbiology and microbial genetics. Specifically, the present invention relates to methods for targeted cloning of DNA fragments from the genome of a donor organism. Further, the present invention refers to a vector, as well as the use of said vector for cloning and/or transferring of a DNA fragment.

Description of the background art

Bacteria and Actinomycetes (or Actinobacteria) in particular are abundant source of biologically active secondary metabolites. A large number of clinically important secondary metabolites are routinely used for treatment as antibiotics, antifungals and inhibitors of a wide range of metabolic or regulatory functions.

Secondary metabolite gene clusters of Actinobacteria such as Actinobacteria of the genus Actinomycetes are large sequences in the genome of these microorganisms which usually contain clustered genetic information for catalytic enzymes, regulatory proteins and auxiliary proteins such as resistance genes, transporters etc. The size of these clusters, especially where very large polyketide synthase genes or non ribosomal peptide synthase genes are encoded, often exceeds the size of DNA fragments which can be efficiently cloned using current molecular biology tools.

Until now, there are several approaches known for cloning gene fragments of different organisms.

For instance, an existing solution to the size limitation of common cloning vectors for E. coli are Cosmids and BAC (Bacterial artificial chromosome) vectors. The former (Cosmids) allow construction of random genome libraries consisting of Cosmid clones harbouring approximately 40kb (kilo base) DNA fragments. When a whole cluster cloning is the target of such methods, additional steps of library screening for Cosmid clones containing parts of the target cluster as well as assembly of the identified clones into a functional whole cluster DNA fragment are necessary. The latter, i.e. the BAC vectors, allow cloning of significantly larger fragments of DNA (more than 300kb DNA fragments have been reported), however the step of screening of a BAC library is still a necessary step when targeting a specific gene cluster.

Further, there is a well-known approach of targeted cloning of DNA fragments based on DNA sequence of the desired DNA fragment (the so-called “plasmid rescue approach”), wherein a plasmid with E. coli replication function is integrated into the vicinity of the targeted cluster in the donor microorganism using homologous recombination with a cloned homologous fragment integrated into the said plasmid. Upon stable integration into the donor genome or donor chromosome, respectively, the complete genomic DNA is digested using restriction endonucleases selected on the basis of the available DNA sequence of the region of the targeted genetic cluster. In the next step, a ligation reaction is set up with the digested genomic DNA under conditions promoting intramolecular ligation (cyclisation) and used to transform an E. coli host. The transformants arising from cyclic DNA fragments containing replication functions and resistance markers delivered with the plasmid can be efficiently selected.

With this regard, WO 2009/01 7692 discloses the cloning and expression of the actinorhodin gene cluster, which is a streptomycetes secondary metabolite biosynthetic gene cluster.
Up to date, the above approaches exhibit significant limitations, e.g. in cloned DNA fragments size (<40kb), which can be stably maintained in E. coli. Further, the known approaches are laborious. So far, there have been no reports of a tool based on a plasmid rescue approach which would facilitate the plasmid rescue method, e.g. in terms of direct transformation of a homologous or heterologous host, such as actinomycetes, without additional modification of the plasmid with the cloned DNA fragment.

Despite the above described known approaches and attempts for improving cloning of desired DNA fragments that e.g. carry gene clusters encoding secondary metabolites, there is still a need for an improved cloning method and improved downstream processes such as heterologous expression of the cloned DNA fragment in a broad range of host cells such as bacteria.

Summary of the invention

The present invention provides the following aspects, subject-matters and preferred embodiments, which respectively taken alone or in combination, contribute to solving the object of the present invention:

(1) A method for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

(a) Transferring a Bacterial Artificial Chromosome (BAC)-vector into the donor organism, wherein said vector comprises
   (a1) an origin of replication;
   (a2) an origin of transfer;
   (a3) a site-specific recombination system containing a functional integrase and an attP site;
   (a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
   (a5) a selection marker,
   wherein said donor organism does not contain a functional attB site
(b) Isolating the genomic DNA from the donor organism;
(c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;
(d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);
(e) Optionally, isolating said BAC-vector from the prokaryotic organism of step (d);
(f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome.

(2) The method according to item (1), wherein the integrase of step (a3) is a 0C31 integrase.

(3) A method for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

(a) Transferring a BAC-vector into the donor organism, wherein said vector comprises
   (a1) an origin of replication;
(a2) an origin of transfer;
(a3) a site-specific recombination system containing an attP site however no functional integrase;
(a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
(a5) a selection marker,

(b) Isolating the genomic DNA from the donor organism;
(c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;
(d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);
(e) Optionally, isolating said BAC-vector from the prokaryotic organism of step (d);
(f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome by action of an integrase the host cell is provided with.

(4) The method according to any of the previous items, wherein the BAC-vector comprises one origin of replication.

(5) The method according to item (3) or (4), wherein the functional integrase of step (f) is a 0C31 integrase.

(6) The method according to any of the previous items, wherein the prokaryotic organism is E. coli.

(7) The method according to any of the previous items, wherein the BAC-vector is a shuttle BAC-vector, preferably an E. coli - Actinomycetes conjugative vector.

(8) The method according to any of the previous items, wherein between any of steps (c) to (f), e.g. between steps (c) and (d), or between (d) and (e), or between (e) and (f), or between (c) and (e), or between (c) and (f), or between (d) and (f), preferably between steps (c) and (f), the BAC-vector from step (c), i.e. the BAC-vector derived from the BAC-vector from step (a), is not modified, preferably no attP-site, attP-site integrase cassette, and/or integrase cassette is introduced into said BAC-vector.

(9) The method according to any of the previous items, further comprising the following steps:
   - after step (a) and prior to step (b): Selecting for the donor organism that contains the BAC-vector incorporated into the donor organism genome; and/or
   - after step (b) and prior to step (c): Cleaving and self-ligation of said isolated genomic DNA.

(10) The method according to any of the previous items, wherein the transfer of the BAC-vector into the donor organism comprises a homologous recombination step.

(11) The method according to any of the previous items, wherein the conjugation of step (f) is mediated by electrocompetent E. coli cells that contain a conjugation helper plasmid.

(12) The method according to any of the previous items, wherein the genomic DNA that is present in the BAC-vector from steps (c) to (f) comprises a DNA fragment from the donor organism.
The method according to item (12), wherein the DNA fragment has a size of from about 5 kb to about 500 kb.

The method according to items (12) or (13), wherein the DNA fragment comprises a whole gene cluster or a portion of a gene cluster, preferably a whole gene cluster, of the donor organism.

The method according to item (14), wherein the whole gene cluster or portion of a gene cluster, preferably the whole gene cluster, encodes one or more gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites.

The method according to items (14) or (15), wherein the whole gene cluster or portion of a gene cluster, preferably the whole gene cluster, comprises the whole clavulinic acid cluster or parts thereof.

The method according to any of the previous items, wherein the donor organism is a bacterium, and preferably is selected from the group consisting of acidobacteria, actinobacteria, bacetroidets, firmicutes, proteobacteria or thermodesulfobacteria;


even more preferably, the donor organism is a bacterium selected from the group consisting of the families Acidobacteriaceae, Actinomycetaceae, Actinopolysporaceae, Corynebacteriaceae, Mycobacteriaceae, Nocardiaceae, Brevibacteriaceae, Microbacteriaceae, Micrococcales, Promicromonosporaceae, Aarobacteraceae, Micromonosporaceae, Nocardioidaceae, Propionibacteriaceae, Actinosynnemataceae, Pseudonocardiae, Streptomycetaceae, Nocardipsoaceae, Streptosporangiaceae, Bifidobacteriaceae, Bacteroidaceae, Flavobacteriaceae, Chlamydiaceae, Bacillaceae, Listeriaceae, Paenibacillaceae, Planococcaceae, Staphylococcaceae, Aerococcaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Clostridiaceae, Eubacteriaceae, Heliohabitaceae, Caulobacteriaceae, Methylobacteriaceae, Rhizobiales.
Xanthobacteraceae, Rhodobacteraceae, Acetobacteraceae, Rhodospirillaceae, Alcaligenaceae, Burkholderiaceae, Comamonadaceae, Neisseriaceae, Myxococcales, Nannocystaceae, Phaselicystidaceae, Pseudomonaceae, Sandaracinaeae, Syntrophaceae, Syntrophobacteraceae, Syntrophorhabdaceae, Campylobacteraeae, Aeromonadaceae, Alteromonadaceae, Pseudoalteromonadaceae, Psychromonadaceae, Shewanellaceae, Enterobacteriaceae, Alcanivoracaceae, Haellaceae, Pseudomonadaceae, Vibrionaceae and Mycoplasmataeae; most preferably, the donor organism is selected from the group consisting of Acidobacteriaceae, Actinomycetaceae, Actinopolysporaceae, Corynebacteriaceae, Nocardioidaceae, Brevibacteriaceae, Micromonosporaceae, Nocardioidaeae, Actinosynemateaeae, Pseudonocardiaceae, Streptomyctaceae, Nocardioidaceae, Enterobacteriaceae, Rhodobacteraceae, Acetobacteraceae, Rhodospirillaceae, Alcaligenaceae, Burkholderiaceae, Comamonadaceae, Myxococcales, Nannocystaceae, Phaselicystidaceae, Pseudomonaceae, Syntrophobacteraceae, Syntrophorhabdaceae, Campylobacteraeae, Aeromonadaceae, Enterobacteriaceae, Alcanivoracaceae and Pseudomonadaceae; and even more preferably, the donor organism is selected from the group consisting of Actinomycetaceae, Actinopolysporaceae, Brevibacteriaceae, Nocardioidaceae, Micromonosporaceae, Actinosynemateaeae, Streptomyctaceae, cardioseae, Pseudonocardiaceae, Bacillaceae, Listeriaceae, Lactobacillaceae, and Leuconostocaceae.

The method according to any of the previous items, wherein the donor organism is preferably selected from the group consisting of Streptomyces ambofaciens, Streptomyces avermitilis, Streptomyces capreolus, Streptomyces carnieostaticus, Streptomyces cervinus, Streptomyces clavuligerus, Streptomyces davawensis, Streptomyces fradiae, Streptomyces griseus, Streptomyces hygroscopicus, Streptomyces lavendulae, Streptomyces lividans, Streptomyces natalensis, Streptomyces noursei, Streptomyces kanamyceticus, Streptomyces nodosum, Streptomyces tsukubaensis, Streptomyces sp., Streptomyces coelicolor, Streptomyces cinnamoneus, Streptomyces platensis, Streptomyces rimosus, Streptomyces spectabilis, Streptomyces verticillus, Streptomyces venezuelae, Streptomyces violaceoniger, Streptomyces violaceorubidus, Actinomycetes bovis, Actinomycetes bowdenii, Actinomycetes canis, Actinomycetes catuli, Actinomycetes coleocanis, Actinomyces eupeusae, Actinomyces funkei, Actinomyces georgiae, Actinomyces hongkongensis, Actinomyces israelii, Actinomyces marinmammalium, Actinomyces meyeri, Actinomyces oricola, Actinomycetes slackii, Actinomycetes streptomyccin, Actinomyces suis, Actinoplanes teichomyceticus, Actinosynemama pretosum, Pseudonocardia autotrophica, Amycolatopsis mediteraneae, Amycolatopsis orientalis, Saccharopolyspora erithareae, Saccharopolyspora hirsuta, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas syringae, Xantomonas oryzae, Burkholderia cenocepacia, Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Mixococcus xantus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus brevis, Lactococcus lactis, Brevibacterium casei, Brevibacterium oxydans, Bilidobacterium bilidum, Bilidobacterium longum, Commononspora sp., Corynebacterium glutamicum, Rhodococcus sp., Serratia marcescens, Rhizobium trifoli, Rhizobium radiobacter, Sporangium cellulosa, Chondromyces croatus, Gluconobacter oxydans, Micromonospora grisea, Micrococcus sp., Micromonospora rhodorangea, Micromonospora zionensis, Micromonospora purpurea, Micromonospora inyoensis, Micromonospora sagamiensis, Palmonospora parontospora, Dactylosporangium matsuzakienae. Nocardia lactamurans, Bacillus colistinus, Planobispora rosea, Streptomyces arenae, Streptomyces antibioticus, Streptomyces kasugaensis, Streptomyces milakaensis, Streptomyces bikinisensis, Streptomyces alboniger, Streptomyces tenebrarius, Bacillus circularis, Streptomyces ribosidificus, Bacillus colistinus, Streptomyces tenjimariensis, and Streptomyces pactum; preferably, the donor organism is Streptomyces clavuligerus.

The method according to any of the previous items, wherein the host cell is selected from the group consisting of Streptomyces, Saccharopolyspora, Nocardia, Pseudonocardia, Amycolatopsis, Actinobacterium, Pseudomonas, Gluconobacter, Xantomonas, Burkholderia, E. coli, Bacillus, Mixococcus, Lactobacillus, Lactococcus,
Bifidobacterium, Comamonas, Corynebacterium, Brevibacterium, Rhodococcus, Azotobacter, Citrobacter, Enterobacter, Clostridium, Klebsiella, Salmonella, Saccharomyces, Zygosacharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Dunahella, Debaryomyces, Mucor, Torulopsis, Serratia, Rhizobium, Zymomonas, Propionibacterium, Acetobacter, Arthrobacter, Ralstonia, Sporangium, Chondromyces, Gluconobacter, Propionibacterium, Aspergillus, Neurospora, Chrysosporium, Thielavia, Neurospora, Aureobasidium, Fibbasidium, Piromyces, Cryptococcus, Acremonium, Tolypocladium, Scytalidium, Schizophyllum, Sporotrichum, Penicillium, Mucor, Fusarium and Trichoderma; preferably the host cell is selected from the group consisting of Streptomyces, Saccharopolyspora, Nocardia, Pseudonocardia, Amycolatopsis, Pseudomonas, Xanthomonas, Burkholderia, Bacillus, Mixoccoccus, Lactobacillus, Lactococcus, Brevibacterium, Comamonas, Corynebacterium, Rhodococcus, Saccharomyces, Pichia, Kluyveromyces, Candida, Serratia, Rhizobium, Sporangium, Chondromyces, Gluconobacter, Aspergillus, Neurospora, Acremonium, Penicillium, Mucor and Escherichia coli; preferably, the host cell belongs to the genus Streptomyces and, more preferably, is selected from the group consisting of Streptomyces clavuligerus, Streptomyces avermitilis, Streptomyces ambofaciens, Streptomyces fradiae, Streptomyces griseus, Streptomyces hygroscopicus, Streptomyces lavendulae, Streptomyces lividans, Streptomyces natalensis, Streptomyces platensis, Streptomyces rimosus, Streptomyces venezuelae, Pseudonocardia autotrophica, Amycolatopsis mediterranei, Amycolatopsis orientalis, Saccharopolyspora erithrea, Saccharopolyspora hirsute, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas syringae, Penicillium citrinum, Penicillium chrysogenum, Xanthomonas oryzae, Burkholderia cenocepacia, Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Mixococcus xanthus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus brevis, Lactococcus lactis, Brevibacterium casei, Brevibacterium oxydans, Bifidobacterium bifidum, Bifidobacterium longum, Comamonas sp., Corynebacterium glutamicum, Rhodococcus sp., Aspergillus nidulans, Aspergillus niger, Aspergillus terreus, Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis, Candida albicans, Candida antarctica, Serratia marescens, Rhizobium trifoli, Rhisobium radiobacter, Sporangium cellulosum, Chondromyces crocatus, Gluconobacter oxydans, Neurospora crassa, Acremonium chrysogenum and Mucor mucor; and most preferably, the host cell is selected from the group consisting of S. clavuligerus, S. lividans, S. avermitilis, and E. coli.

(20) The method according to any of the previous items, wherein the origin of replication is ori S.

(21) The method according to any of the previous items, wherein the origin of transfer is ori T.

(22) The method according to any of the previous items, wherein the site-specific recombination system is a 0C31 attP-int recombination system.

(23) The method according to any of the previous items, wherein the method is a plasmid rescue method.

(24) A vector comprising

1. an origin of replication;
2. an origin of transfer;
3. a site-specific recombination system containing a functional integrase and an attP site;
4. a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism; and
5. a selection marker.

(25) The vector according to item (24), wherein the integrase is a 0C31 integrase, and/or wherein the donor organism, which preferably does not contain a functional attB site, is a donor organism as defined in item (17) or (18).
(26) A vector comprising

(1) an origin of replication;
(2) an origin of transfer;
(3) a site-specific recombination system containing an attP site however no functional integrase; and
(4) a selection marker.

In a preferred embodiment, the vector of item (26) comprises SEQ ID NO: 6; more preferred, the vector of item (26) has a sequence according to SEQ ID NO: 6.

(27) The vector according to any of items (24) to (26), wherein said vector comprises one origin of replication.

(28) The vector according to item (26) or (27), wherein said vector further comprises a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism.

(29) The vector according to any of items (24) to (28), wherein said vector is a BAC-vector suitable for cloning and/or transfer of a DNA fragment.

(30) The vector according to any of items (24) to (29), wherein the BAC-vector is a shuttle BAC-vector, preferably an E. coli - Actinomycetes conjugative vector.

(31) The vector according to any of items (24) to (30), wherein said vector further comprises a DNA fragment that is intended to be cloned or transferred, preferably said DNA fragment is as defined in items (13) to (16)

(32) The vector according to any of items (24) to (31), wherein the origin of replication is ori S, and/or the origin of transfer is ori T.

(33) Use of a vector of any of items (24) to (32) for cloning and/or transferring of a DNA fragment.

(34) Use according to item (33), wherein the DNA fragment is as defined in items (13) to (16).

(35) A donor organism, comprising a BAC-vector as defined in items (1) (a1) - (a5), or as defined in any one of items (24) or (25), or (27) to (32).

(36) The donor organism according to item (35), wherein said donor organism does not contain a functional attB site.

(37) The donor organism according to item (35) or (36), wherein the BAC-vector comprises a OCS31 integrase.

(38) A donor organism, comprising a BAC-vector as defined in item (3) (a1) to (a5), or as defined in any one of items (26) to (32).

(39) The donor organism according to any of items (35) to (38), wherein the BAC-vector is a shuttle BAC-vector, preferably an E. coli - Actinomycetes conjugative vector.
(40) The donor organism according to any of items (35) to (39), wherein the origin of replication of the BAC-vector is ori S, and/or wherein the origin of transfer is ori T.

(41) The donor organism according to any of items (35) to (40), wherein the site-specific recombination system is a ΦC31 attP-int recombination system.

(42) The donor organism according to any of items (35) to (41), wherein the donor organism is as defined in items (17) or (18).

(43) An expression system, comprising

1. a host cell, and
2. a BAC-vector, wherein said BAC-vector comprises
   i) an origin of replication;
   ii) an origin of transfer;
   iii) a site-specific recombination system containing an attP site and a functional integrase;
   iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and
   v) a selection marker.

(44) An expression system, comprising

1. a host cell, wherein said host cell is provided with a functional integrase, and
2. a BAC-vector, wherein said BAC-vector comprises
   i) an origin of replication;
   ii) an origin of transfer;
   iii) a site-specific recombination system containing an attP site however no functional integrase;
   iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and
   v) a selection marker.

(45) The expression system according to item (43) or (44), wherein the BAC-vector comprises one origin of replication.

(46) The expression system according to any of items (43) to (45), wherein the host cell is as defined in item (19), the origin of replication is ori S, the origin of transfer is ori T, the site-specific recombination system is ΦC31 attP-int recombination system, and/or the BAC-vector is as defined in items (29) or (30).

(47) Use of the expression system according to any of items (43) to (46) for cloning and/or transfer of the DNA fragment, and/or for expression of said DNA fragment.

(48) A vector comprising the sequence according to SEQ ID NO: 3.

(49) A vector comprising the sequence according to SEQ ID NO: 53.

(50) The vector according to item (48) or (49), additionally comprising a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism.
The vector according to item (50), additionally comprising a DNA fragment as defined in any of items (13) to (16).

Use of a vector according to any of items (48) to (51) for cloning and/or transfer of a DNA fragment, and/or for expression of said DNA fragment.

Definitions of terms as used within the meaning of the present invention

As used herein, the term "donor organism" refers to the organism that comprises the DNA fragment(s) in its genome that is intended for being cloned and/or transferred e.g. into a host cell. Within the meaning of the present invention, such a donor organism is any microorganism which accepts foreign DNA via the conjugal transfer from E. coli, and preferably integrates said DNA via homologous integration, and does not support replication of the BAC replicon.

Herein, the term "host cell" denotes the organism that finally receives the cloned DNA fragment of the donor organism. In one embodiment according to the present invention, the cloned DNA fragment is multiplicated (e.g. duplicated), or amplified, respectively, in the host cell. It is also possible that the cloned DNA fragment is expressed in a host organism that, naturally, does not express said DNA fragment.

Within the meaning of the present invention, the term "DNA fragment" refers to a piece of DNA that has a size of desired length, suitably of at least about 5 kb, preferably at least about 10 kb, more preferably at least about 20 or 30 kb, even more preferably at least about 40 or 50 kb. The upper size limit of said piece of DNA can be chosen as desired, suitably is about 300 kb, preferably about 350 or 400 kb. In some embodiments, the upper size limit is even higher, such as 450 or 500 kb. Further within the meaning of the present invention, the DNA fragment originates from the genome of a donor organism and preferably comprises a whole gene cluster or a portion of a gene cluster of said donor organism.

The term "about" defines a deviation of 20%, preferably of 10%, more preferably of 7% and even more preferably of 3% of a given value.

A "vector" is a replicon, such as plasmid, phage, bacterial artificial chromosome (BAC) or cosmid, to which another DNA segment (e.g. a foreign gene) may be incorporated so as to bring about the replication of the attached segment, resulting in expression of the introduced sequence. Vectors may comprise a promoter and one or more control elements (e.g., enhancer elements) that are heterologous to the introduced DNA but are recognized and used by the host cell. Alternatively, the sequence that is introduced into the vector retains its natural promoter that may be recognized and expressed by the host cell (Bormann et al., J. Bacteriol1996;178:1216-1218). A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication within a cell, i.e., capable of replication under its own control.

A BAC-vector within the meaning of the present invention denotes a cloning vector derived from a bacterial chromosome into which a large genomic DNA fragment can be inserted, typically up to 400 kb. BAC-vectors are F-plasmid (F factor) derived. Further, BAC-vectors can comprise a prokaryotic F factor partitioning system, which ensures that the daughter cells inherit a copy of the parental plasmid.

A vector can also be a Bacterial Artificial Chromosome (BAC) shuttle vector. This is a vector that permits conjugation between e.g., Streptomyces and E. coli. As known to a skilled person, a common way to insert one segment of DNA into another segment of DNA involves the use of specific enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or
segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA.

A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can be readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Vector constructs may be produced using conventional molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); F.M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Within the meaning of the present invention, the term "BAC-vector derived from..." (e.g. derived from a previous (or predecessor) BAC-vector) defines a BAC-vector that comprises most of the regulatory elements of the previous BAC-vector from which it is derived, however does not completely correspond to said previous BAC-vector. For instance, a BAC-vector that is derived from a previous BAC-vector still comprises regulatory elements such as origin of replication, origin of transfer, a site-specific recombination system (e.g. containing an integrase and an attP site, or containing an attL site however no functional integrase), and/or a selection marker. Further within the meaning of the present invention, the BAC-vector that is derived from a previous BAC-vector comprises a nucleic acid (e.g. DNA) fragment that comprises a whole gene cluster or a portion of a gene cluster, preferably a whole gene cluster, which gene cluster, in turn, comprises a nucleic acid (e.g. DNA) sequence that is substantially homologous or completely homologous to a nucleic acid (e.g. DNA) sequence that is present in the previous BAC-vector. Said nucleic acid sequence that is present in the previous BAC-vector in general is of a length that allows for homologous recombination, and, in general, has a suitable size such as about 1kb-2kb. The above gene cluster represents the gene cluster that is intended to be cloned and/or transferred.

Within the meaning of the present invention, the term "plasmid rescue method" defines a method that can be used for isolating (chromosomal) DNA adjacent to an inserted piece of DNA in various organisms, such the donor organisms as defined herein elsewhere. In other words, the "plasmid rescue method" provides for a means of isolating (i.e. removing of a material of interest such as a DNA fragment or a genetic cluster form its natural environment) or recovering a gene cluster that lies adjacent to the integration site, such that it is free of any other contaminating genetic or cellular material.

The term "gene cluster" denotes any group of two or more closely linked genes that encode for the same or similar products. Secondary metabolites often play an important role in plant defense against herbivory and other interspecies defenses. In bacteria, secondary metabolites have a primary role in the struggle for nutrients and habitat in a complex microbial environment. Consequently, most secondary metabolites have biological activity against competing bacteria, fungi or other organisms. Some secondary metabolites are acting as inhibitors of competitor's nutrient uptake enzymes, others directly displaying antibacterial or antifungal activity, others have activity which counters competitor's defence mechanisms and yet others counter competitor's offence mechanism. It is well known that secondary metabolites show incredible wealth of diversity in terms of chemical characteristics. Therefore, humans
use some secondary metabolites as medicines, flavorings, and recreational drugs. Secondary metabolites can be divided in the following categories: Small "small molecules", such as beta-lactams, alkaloids, terpenoids, glycosides, natural phenols, phenazines, bifeny1s and dibenzofurans; big "small molecules", produced by large, modular, "molecular factories", such as polyketides, complex glycosides, nonribosomal peptides, and hybrids of the above three; and non-"small molecules" - DNA, RNA, ribosome, or polysaccharide "classical" biopolymers, such as ribosomal peptides. Preferably, the secondary metabolites according to the present invention are small "small molecules", such as beta-lactam.

In order to propagate a vector in a host cell, it may contain one or more "origins of replication" sites (often also termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Accordingly, the term "origin of replication" or "ori" refers to a nucleic acid sequence that initiates nucleic acid replication.


"Ori T" refers to an "origin of transfer" that permits the transfer of the vector from one bacterial cell to another.

The "origin of transfer" (e. g. ori T) represents the site on the vector where the transfer process is initiated. It is also defined genetically as the region required in cis to the DNA that is to be transferred. Conjugation-specific DNA replication is initiated within the ori T region which also encodes plasmid transfer factors.

An enzyme called "integrase" recognizes two att sites, joins the two DNA molecules and catalyzes a DNA double-strand breakage. A rejoining event results in the integration of one of the DNA molecules into the other DNA of the recipient cell. (N. D. Grindley, K. L. Whiteson, P. A. Rice, 2006. Annu. Rev. Biochem. 75, 567-605.)

The integrases ΦΒΤ 1 or OC31, in combination with attP or attB, allow for site-specific recombination, which permits site-specific integration of the vector into the respective attB sites of the recipient cell's chromosome. (Gregory, M.A., Till, R., and Smith, M.C.M. 2003. J Bacterid. 185:5320-5323; GenBank Accession Number AJ550940). The site-specific recombination is facilitated by an "att" site, which is a site that has nucleic acid identity or similarity which facilitates said site-specific recombination. The "attB" site refers to the attachment site on the bacterial cell chromosome, and the "attP" site refers to the attachment site on the bacteriophage. The sequence of att sites is different and specific for distinct integrases such as ΦΒΤ 1 or OC31, therefore integration into the recipient genome is at different loci.

The term "functional" defines that the respective matter exhibits its natural function. For instance, a functional attB or attP site is an attB or attP site that mediates, in combination with a functional integrase, a site-specific recombination. An attB/P site or integrase that is not functional (any more) can for instance have lost its respective function due to genetic modification, such as gene deletion, or gene disruption.

A "site-specific recombination" refers to a recombination process between two DNA molecules that occurs at unique sites - the integration site - of each molecule which are generally 20 to 30 bases long, and that are called attachment (att) sites. A specialised enzyme called integrase recognizes the two att sites, finally resulting in the integration of one of the DNA molecules into the other DNA of the recipient cell (N. D. Grindley, K. L. Whiteson, P. A. Rice, 2006. Annu. Rev. Biochem. 75, 567-605.).
Within the meaning of the present invention, two DNA sequences are “substantially homologous” if they are able to mediate a homologous recombination. This may for instance the case when at least about 80%, preferably at least about 90% or 95%, more preferably at least about 97% or 98% of the nucleotides match over a defined length of the DNA sequences, as determined by sequence comparison algorithms known to a person skilled in the art, such as BLASTA, FASTA, DNA Strider, and so on. It is also possible that two DNA sequences exhibit 100% match of the nucleotides over a defined length. In this case, the two DNA sequences are homologous to each other. Within the meaning of the present invention, a DNA sequence in the BAC-vector is substantially homologous, or homologous, to a desired target integration site in the donor organism.

The term “homologous recombination” refers to a type of genetic recombination, a process of physical rearrangement occurring between two different strands of DNA molecules. Homologous recombination involves the alignment of identical or similar sequences, a crossover between the aligned homologous DNA strands of the two molecules, and breaking and repair of the DNA to produce an exchange of material between the strands. Homologous recombination is distinguished from other types of recombination. For example, “site specific recombination”, as exemplified by invertible elements, resolvases, and some phage integration events are examples of non-homologous recombination. Though in many cases identical or similar sequences are required at the two recombining sites, the sequences are short, distinguishing them from the longer stretches (hundreds of base pairs) used in homologous recombination. (J Rubnitz and S Subramani. 1984, Mol Cell Biol. 4: 2253-2258).

The term “selecting” refers to the identification and isolation of a recipient cell such as the donor organism or host organism (or donor cell/donor strain or host cell/strain, respectively) or a further prokaryotic organism (such as E. coli) that contains the vector of interest (such as a BAC-vector as defined herein, e.g. a BAC-vector containing the cloned gene cluster). Transformed microorganisms, that is, those containing recombinant molecules such as a vector or plasmid, may be selected with a variety of positive and/or negative selection methods or markers. For instance, a positive selection marker can be a gene that allows growth in the absence of an essential nutrient, such as an amino acid. A variety of suitable positive/negative selection pairs are available in the art. For example, various amino acid analogs known in the art could be used as a negative selection, while growth on minimal media (relative to the amino acid analog) could be used as a positive selection. Visually detectable markers are also suitable for being used in the present invention, and may be positively and negatively selected and/or screened using technologies such as fluorescence activated cell sorting (FACS) or microfluidics. Examples of detectable markers include various enzymes, prosthetic groups, fluoroscent markers, luminescent markers, bioluminescent markers, and the like. Examples of suitable fluoroscent proteins include, but are not limited to, yellow fluorescent protein (YFP), green fluorescence protein (GFP), cyan fluorescence protein (CFP), umbelliflone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrín and the like. Examples of suitable bioluminescent markers include, but are not limited to, luciferase (e.g., bacterial, firefly, click beetle and the like), luciferin, aequorin and the like. Examples of suitable enzyme systems having visually detectable signals include, but are not limited to, galactosidases, glucorinidases, phosphatases, peroxidas, cholinesterases and the like. In other aspects, the positive selection marker is a gene that confers resistance to a compound, which would be lethal to the cell in the absence of the gene. For example, a cell expressing an antibiotic resistance gene would survive in the presence of an antibiotic, while a cell lacking the gene would not. For instance, the presence of a tetracycline resistance gene could be positively selected for in the presence of tetracycline, and negatively selected against in the presence of fusaric acid. Suitable antibiotic resistance genes include, but are not limited to, genes such as ampicillin-resistance gene, neomycin-resistance gene, blasticidin-resistance gene, hygromycin-resistance gene, puromycin-resistance gene, chloramphenicol-resistance gene, apramycin-resistance gene and the like. In certain aspects, the negative selection marker is a gene that is lethal to the target cell in the presence of a particular substrate. For example, the thyA gene is lethal in the presence of trimethoprim. Accordingly, cells that grow in the presence trimethoprim do not
express the thyA gene. Negative selection markers include, but are not limited to, genes such as thyA, sacB, gnd, gapC, zwJ, talA, talB, coda, ppc, gdhA, pgI, Jbp, pykA, cit, acs, edd, icdA, groEL, secA and the like.

Within the meaning of the present invention, the term "isolating" refers to the removal of a material of interest from its environment (e.g. from an environment into which it has been placed). For instance, the material of interest can be genomic DNA, which is isolated from an organism, e.g. from a donor organism as defined herein, or from a host organism as defined herein, such as E. coli.

Further within the meaning of the present invention the term "conjugation" refers to the direct transfer of nucleic acid from one prokaryotic cell to another via direct contact of cells. The cell that carries the transferred DNA after conjugation is termed "exconjugant" or "exconjugant". Thus, a "conjugative vector" (for example, pSBAC) is a vector that contains a nucleic acid of interest, whereby such nucleic acid is directly transferred (i.e. the passing of a nucleic acid sequence from one cell to another without isolation of the sequence) from one cell to another via direct contact between the cell containing the vector and a recipient cell to which the nucleic acid is transferred following direct contact of the two cells. As used herein, the term "conjugative transfer" refers to the temporary union of two bacterial cells during which one cell transfers part or all of its genetic material to the other.

"Secondary metabolites" are organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms. Unlike primary metabolites, absence of secondary metabolites only results in mild impairment for the organisms such as: lowered survivability/fecundity, aesthetic differences, or else no change in phenotype at all.

Within the meaning of the present invention, the term "expression system" denotes a system that is specifically designed for the production of a gene product of choice. For instance, in the present invention the expression system is able to produce the gene product of the DNA fragment (or gene cluster, or part thereof) that previously has been intended for being cloned and/or transferred.

Within the meaning of the present invention, the term "attB deletion strain" denotes a (donor) strain that does not comprise a functional attB site. It is understood that some organisms may lack the attB site in their genome in their native state. Therefore, within the meaning of the present invention either situation is allowed to carry out the cloning of the gene clusters, i.e. using a donor organism that naturally lacks the attB site, and using a donor organism that had to be treated in a specific way (e.g. by using molecular cloning methods) in order to delete the attB site. In general, the integrase is not present in the donor strain.

A "prokaryotic F factor partitioning system" refers to an active positioning process that ensures proper segregation and faithful distribution of daughter F factors at cell division. This partitioning system contains three functionally distinct regions: two of them (sopA and sop B) encode gene products that act in trans, whereas the third region (sopC) functions in cis.

An "F factor" or "prokaryotic F factor" refers to a fertility factor found in prokaryotes. It is a small piece of episomal DNA that enables bacteria to mediate conjugation with other bacteria. The F factor "partitioning system" refers to the system that ensures that both daughter cells inherit a copy of the parental plasmid.

Detailed description of the invention
The present invention is now described in more detail by preferred embodiments and examples, which are however presented for illustrative purpose only and shall not be understood as limiting the scope of the present invention in any way.

The present invention provides an improved method for targeted cloning and/or transferring of DNA fragments from a donor organism into a host cell. This is achieved by (I) a method comprising the step of transferring a BAC-vector into the donor organism wherein said vector contains an integrase and an attP site and is introduced into a donor organism that does not contain a functional attB site.

Further, this is also achieved by (II) applying a method that comprises the step of transferring a BAC-vector into a donor organism, wherein said vector comprises a site-specific recombination system that contains an attP site however, no functional integrase and said donor organism may or may not have a functional attB site.

In prior art methods it has often been necessary in targeted cloning methods to modify the BAC-DNA plasmid that comprises the cloned DNA fragment and that is to be transferred into a host cell. Such modification can for instance be the introduction of attP-integrase cassettes: in case said cassette is introduced into the BAC-vector prior to the plasmid rescue attempt, the vector would preferably integrate into the attB site of the genome and not to the desired targeted locus. Hence, in prior art methods, modifications such as the introduction of attP-integrase cassettes often have to be carried out on the BAC-DNA plasmid that comprises the cloned DNA fragment and that is to be transferred into a host cell. Such modifications are difficult as standard molecular methods do not apply, and, instead, are laborious, specialized and lengthy. Further, any additional propagation of the BAC-DNA plasmid that comprises the cloned DNA fragment, especially when the second inducible multicopy repilon (e.g. ori V) is present in the BAC-vector, poses a risk of unwanted rearrangements in the BAC-DNA plasmid which may be caused by recombination events, faulty segregation etc.

It has been surprisingly found that by applying the method according to the present invention, an improvement of the presently known prior art methods for cloning and/or transfer of DNA fragments as defined herein and/or for expression of said DNA fragments, can be achieved. In particular, an improvement in terms of time required for cloning and/or transferring and/or expressing DNA fragments can be achieved, as the desired targeted gene cluster (which is comprised in a DNA fragment) can be cloned in a targeted approach and directly transferred to the host cell or host organism, respectively. "Directly transferred" in the context of the present invention denotes that there is no need to modify the rescued BAC-vector: Thus, a modification of the BAC-DNA plasmid that comprises the cloned DNA fragment and that is to be transferred into a host cell (e.g. a heterologous or homologous actinomycyes host) can be avoided. This is reached by two approaches: One approach (I) is to prepare an attB deletion strain of the organism which is the donor of the DNA fragment to be cloned (donor organism). The BAC-vector, which comprises a functional attP site, a functional integrase and a DNA sequence that allows for homologous recombination with a desired target site of the genome of the donor organism, is transferred into such an attB deletion strain. The BAC-vector integrates into the desired target site on the genomic DNA of the donor organism via homologous recombination. Then, the genomic DNA comprising the desired DNA fragment from the donor which is to be cloned and/or expressed, is isolated from the donor organism, followed by a transfer of said genomic DNA, which is present in a BAC-vector, into a prokaryotic organism. This BAC-vector is then introduced into a host cell, whereupon said BAC-vector integrates into the host cell genome. The integration into the host cell's genome is mediated via attP/attB site and integrase. By applying approach (I), large clusters can be cloned in a targeted approach and directly transferred to the host cell or host organism, respectively. The same approach applies in case the donor organism does not contain an attB site in its genome already by nature. In this case, a donor organism that does not contain an attB site in its genome by nature, i.e. naturally, is provided.
The second approach (II) is to use a BAC-vector without the integrase gene however still harbouring the integrase plasmid binding site, the so-called attP site. In this case, the BAC-vector comprising the desired DNA fragment of the donor organism is integrated into the host cell's genome by action of an integrase the host cell is provided with. The integrase that is needed for attP/attB site directed integration of the BAC-vector into the host cell's genome can for instance be provided in trans by a helper plasmid. This latter approach additionally has the advantage that it is even more improved compared to approach (I), as it is for instance more universal as no deletion donor strain has to be prepared.

Further, the present invention can advantageously be used for genome sequence based, targeted cloning of large DNA fragments that originate for instance from actinomycetes genomes, with the purpose of amplification or heterologous expression of complete genetic clusters in a broad range of host cells, such as bacteria. Hence, the invention is useful in the fields of (1) drug discovery, e.g. expression of silent secondary metabolite genetic clusters in heterologous host (genome mining); (2) synthetic biology, e.g. combinatorial assembly of biosynthetic pathways from distinct organisms to yield heterogenous or artificial molecules; (3) process improvement, e.g. heterologous expression of gene clusters where the parent host is inappropriate for industrial production processes or amplification of whole genetic clusters where higher productivities are desired.

Thus, the present invention relates to a method (I), preferably a plasmid rescue method (I), for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

(a) Transferring a Bacterial Artificial Chromosome (BAC)-vector into the donor organism, wherein said vector comprises
   (a1) an origin of replication, preferably ori S (ori 2);
   (a2) an origin of transfer, preferably ori T;
   (a3) a site-specific recombination system containing a functional integrase and an attP site;
   (a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
   (a5) a selection marker:
   wherein said donor organism does not contain a functional attB site,

(b) Isolating the genomic DNA from the donor organism;

(c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;

(d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);

(e) Optionally, isolating the BAC-vector from the prokaryotic organism of step (d);

(f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome.

Step (e), i.e. the isolation of the BAC-DNA from the prokaryotic organism of step (d), is optional, as it is also possible to directly use the prokaryotic organism (such as E. coli) with the BAC-vector for conjugal transfer to a host organism e.g. by applying a suitable "three parental conjugal transfer protocol" which is known to a person skilled in the art, or by additionally transforming the BAC carrying prokaryotic organism by a pUZ type conjugation promoting plasmid.
Moreover, the present invention further relates to a method (II), preferably a plasmid rescue method (II), for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

(a) Transferring a BAC-vector into the donor organism, wherein said vector comprises
   (a1) an origin of replication, preferably ori S (ori2);
   (a2) an origin of transfer, preferably ori T;
   (a3) a site-specific recombination system containing an attP site however no functional integrase;
   (a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
   (a5) a selection marker;
(b) Isolating the genomic DNA from the donor organism;
(c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;
(d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);
(e) Optionally, isolating said BAC-vector from the prokaryotic organism of step (d);
(f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome by action of an integrase the host cell is provided with.

Moreover, by applying the method according to the present invention, DNA fragments having a very large size can be stably maintained in a prokaryotic organism such as E. coli, and, if desired, further integrated in a host cell genome, and e.g. expressed.

As already indicated above, by using the inventive methods, a modification of the BAC-vector of step (c) with the cloned DNA fragment can be avoided. This is a great advantage since molecular modification of DNA larger than about 30 kb is difficult as standard molecular methods do not apply, and, instead, laborious, specialized and lengthy procedures have to be applied. Further, by applying the inventive methods, there is more freedom e.g. with regard to the planned cloning strategy.

In a preferred embodiment, the vectors according to the present invention comprise only one origin of replication. In prior art methods, in general the use of a second (multicopy) ori has been applied in order to improve isolation yields of BAC DNA or in order to increase the efficiency of conjugal transfer. However, at the same time, upon induction of such multicopy mode of BAC-vectors, there exists a danger of losing the most important property of BAC-vectors, i.e. the stable maintenance of large DNA fragments. Within the meaning of the present invention, it has been unexpectedly found that even by using only one origin of replication, surprisingly a sufficient and satisfactory e.g. conjugal transfer of the BAC-vectors from E. coli to a host cell can be obtained, while at the same time the stable maintenance of DNA fragments of larger size, i.e. of a size as indicated elsewhere herein, can be obtained.

In a further preferred embodiment of the present invention, the integrase 0C31 is used. This means that in method (I), the site-specific recombination system in step (a3) contains a 0C31 integrase, and in method (II), in step (f) the integrase the host cell is provided with is a 0C31 integrase. By using a 0C31 integrase, an improved transfer/integration frequency can be obtained compared to the transfer/integration frequency obtained when using other integrases, such as ΦBT1 integrase.
In a first step, a BAC-vector comprising one origin of replication, an origin of transfer, a site-specific recombination system containing an integrase and an attP site (method (i)) or containing an attP site however no functional integrase (method (ii)), and a DNA sequence that allows for homologous recombination with a corresponding DNA sequence being present in the genomic DNA of the donor organism, is transferred into said donor organism. In order to allow for homologous recombination with a defined target sequence (that is present in the donor organism), the DNA sequence is substantially homologous or homologous to the desired, defined target sequence, e.g. the desired target integration site in the genome of the donor organism. Preferably, the desired target integration site in the genome of the donor organism is located adjacent to the DNA fragments (preferably gene cluster(s)) that are intended for being cloned. The presence of DNA sequences in the BAC-vector that are substantially homologous or homologous to said integration site in the genome of the donor organism ensures that upon homologous recombination the BAC-vector integrates in said desired target integration site.

In a preferred embodiment, the BAC-vector additionally comprises a prokaryotic F factor partitioning system. This system ensures that the daughter cells inherit a copy of the parental plasmid.

In general, a donor organism can be any suitable organism that is known to a skilled person and that comprises in its genome the DNA fragment(s) that are desired to be cloned. Within the meaning of the present invention, the donor organism is any microorganism which accepts foreign DNA via the conjugal transfer from E. coli, and preferably integrates said DNA via homologous integration, and does not support replication of the BAC replicon. In a preferred embodiment of the present invention, such a donor organism can for instance be selected from the group consisting of bacteria (gram positive and gram negative bacteria) archaea, and eukaryota (e.g. yeasts, plants, and the like). In one embodiment of the present invention, the donor organism is a bacterium, and preferably is selected from the group consisting of acidobacteria, actinobacteria, bacetoidetes, firmicutes, proteobacteria or thermodesulfobacteria; more preferably, the donor organism is selected from the group consisting of the family Acidobacteriaceae, Actinomycetaceae, Actinopolysporaceae, Corynebacteriaceae, Gordoniaceae, Mycobacteriaceae, Nocardiaceae, Brevibacteriaceae, Cellulomonadaceae, Demequiniaceae, Microbacteriaceae, Micrococcaeae, Promicromonosporaceae, Aerobacteraceae, Micromonosporaceae, Nocardioidaceae, Propionibacteriaceae, Actinosynnemataceae, Pseudonocardiaeae, Streptomycetaceae, Nocardiopsaceae, Streptosporangiacaeae, Thermomonosporaceae, Bifidobacteriaceae, Bacteroidiaceae, Marinilabiliaceae, Morphyromonadaceae, Cyclobacteriaceae, Cytophagaceae, Flammewirgaceae, Rhodothermaceae, Blattabacteriaceae, Cryomorphaceae, Flavobacteriaceae, Schleiferiaceae, Chitinophagaceae, Saprosiraceae, Sphingobacteriaceae, Chlamydiaceae, Parachlamydiaceae, Simkaniaceae, Waddlaciaceae, Bacillaceae, Listeriaceae, Paecilabicaceae, Planococcaceae, Staphylococcaceae, Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Clostridiaceae, Eubacteriaceae, Helicobacteriaceae, Peptococcaceae, Fluminoceccaceae, Caulobacteriaceae, Hyphomonoaceae, Bradyrhizobiaceae, Brucellaceae, Hypomicrobiaceae, Methylobacteriaceae, Rhizobiaceae, Xanthobacteraceae, Rhodobacteraceae, Acetobacteraceae, Rhodospirillaceae, Alcaligenaceae, Burkholderiaceae, Comamonadaceae, Oxalobacteraceae, Neisseriaceae, Spirillaceae, Rhodocyclaceae, Desulfovibacteraceae, Cystobacteraceae, Myxococcaceae, Nannocystaceae, Phaselicyctidaceae, Polyangiaceae, Sandaracinaceae, Syntrophaceae, Syntrophobacteraceae, Syntrophorhabdaceae, Campylobacteraceae, Helicobacteraceae, Acidithiobacillaceae, Aeromonadaceae, Succinivibrionaceae, Alteromonadaceae, Celerinatimominadaceae, Colwelliaceae, Ferrimonadaceae, Idiomarinaeeae, Moritellaceae, Pseudomonadonadaceae, Psychromonadaceae, Shewanellaceae, Chromatiaceae, Ectothiorhodospiraceae, Granuloscoceaceae, Enterobacteriaceae, Coxillaceae, Legionellaceae, Alcanivoraceae, Halallaceae, Halomonadaceae, Litoricolaeeae, Pasteurellaceae, Moraxellaceae, Pseudomonadaceae, Vibrionaceae, Nevskiaceae, Sinobacteraceae, thomonadaceae, Brachyspiraceae, Brevinemataceae, Leptospiraceae, Spirochaetaceae, Haloplasmataceae and Mycoplasmataceae;
even more preferably, the donor organism is a bacterium selected from the group consisting of the families Acidobacteriaceae, Actinomycetaceae, Actinopolysporaceae, Corynebacteriaceae, Mycobacteriaceae, Nocardiaceae, Brevibacteriaceae, Microbacteriaceae, Micrococccaceae, Promicromonosporaceae, Arobacteraceae, Micromonosporaceae, Nocardioidaceae, Propionibacteriaceae, Actinosynnemataceae, Pseudonocardiaceae, Streptomyctaceae, Nocardiopsisaceae, Streptosporangiaceae, Bifidobacteriaceae, Bacteroidaceae, Flavobacteriaceae, Chlamydiaceae, Bacillaceae, Listeriaceae, Paenibacillaceae, Planococcaceae, Staphylococcaceae, Aerococcaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Clostridiaceae, Eubacteriaceae, Helibacteriaceae, Caulobacteraceae, Methylobacteriaceae, Rhizobiaceae, Xanthobacteraceae, Rhodobacteraceae, Acetobacteraceae, Rhodospirillaceae, Alcaligenaceae, Burkholderiaceae, Comamonadaceae, Neisseriaceae, Mycococcaceae, Nannocystaceae, Phaselicyctidaceae, Polyangiaceae, Sandaracinaceae, Syntrophaceae, Syntrophobacteraceae, Syntrophorhabdaceae, Campylobacteraceae, Aeromonadaceae, Alteromonadaceae, Pseudoalteromonadaceae, Psychromonadaceae, Shewanellaceae, Enterobacteriaceae, Alcanivoracaceae, Hahellaceae, Pseudomonadaceae, Vibrionaceae and Mycoplasmataceae; most preferably, the donor organism is selected from the group consisting of Acidobacteriaceae, Actinomycetaceae, Actinopolysporaceae, Corynebacteriaceae, Nocardiaceae, Streptomyctaceae, Micromonosporaceae, Nocardioidaceae, Actinosynnemataceae, Pseudonocardiaceae, Stethopteryctaceae, Nocardiopsisaceae, Streptosporangiaceae, Bacillaceae, Listeriaceae, Lactobacillaceae, Leuconostocaceae, Eubacteriaceae, Xanthobacteraceae, Rhodobacteraceae, Acetobacteraceae, Rhodospirillaceae, Alcaligenaceae, Burkholderiaceae, Comamonadaceae, Mycococcaceae, Nannocystaceae, Phaselicyctidaceae, Polyangiaceae, Syntrophobacteraceae, Syntrophorhabdaceae, Campylobacteraceae, Aeromonadaceae, Enterobacteriaceae, Alcanivoracaceae and Pseudomonadaceae; and

In a further preferred embodiment, the donor organism is selected from the group consisting of Streptomyces ambofaciens, Streptomyces avermitilis, Streptomyces capreolus, Streptomyces carinostaticus, Streptomyces cervinus, Streptomyces clavuligerus, Streptomyces davawensis, Streptomyces fradiae, Streptomyces griseus, Streptomyces hygroscopicus, Streptomyces lavendulæ, Streptomyces lividus, Streptomyces natalensis, Streptomyces noursei, Streptomyces kanamycticus, Streptomyces nodosum, Streptomyces tsukubaensis, Streptomyces sp., Streptomyces coelicolor, Streptomyces cinnamonicus, Streptomyces platensis, Streptomyces rimosus, Streptomyces spectabilis, Streptomyces verticillus, Streptomyces venezuelae, Streptomyces violaceoniger, Streptomyces violaceoruber, Streptomyces mobaraensis, Streptomyces peuceticus, Streptomyces coeruleorubidus, Actinomyces bovis, Actinomyces boerdenii, Actinomyces canis, Actinomyces catuli, Actinomyces colecanis, Actinomyces europaæus, Actinomyces funkei, Actinomyces georgiae, Actinomyces hongkongensis, Actinomyces israeli, Actinomyces marimammalium, Actinomyces meyeri, Actinomyces orica, Actinomyces slackii, Actinomyces streptomycin!, Actinomyces suis, Actinoplanes teichomyceticus, Actinosynnema pretiosum, Pseudonocardia autotrophica, Amycolatopsis mediterranei, Amycolatopsis orientalis, Saccharopolyspora errhathae, Saccharophyllospora hirsuta, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas syringae, Xantomonas oryseae, Burkholderia cenocepacia, Bacillus subtigris, Bacillus licheniformis, Bacillus megaterium, Mixococcus xanthus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus brevis, Lactococcus lactis, Brevibacterium casei, Brevibacterium oxydans, Bifidobacterium bifidum, Bifidobacterium longum, Commononas sp., Corynebacterium glutamicum, Rhodococcus sp., Serratia marcescens, Rhizobium trifoli, Rhizobium radiobacter, Sporangium cellulosum, Chondromyces crocatus, Gluconobacter oxydans, Micromonospora grisea, Micrococcus sp., Micromonospora rhodorangia, Micromonospora zionensis, Micromonospora purpurea, Micromonospora inyens, Micromonospora sagamiensis, Palmonospora parontospora, Dactylosporangium matsuzakiense, Nocardia lactamurans, Bacillus
colistinus, Planobispora rosea, Streptomyces arenae, Streptomyces antibioticus, Streptomyces kasugaensis, Streptomyces mitakaensis, Streptomyces bikiniensis, Streptomyces alboniger, Streptomyces tenebrarius, Bacillus circulars, Streptomyces ribosidificus, Bacillus colistinus, Streptomyces tenjimariensis, and Streptomyces pactum; preferably, the donor organism is *Streptomyces clavuligerus*.

DNA fragments in the genome of the donor organism preferably contain (a) gene cluster(s). In a preferred embodiment of the present invention, said DNA fragments or gene clusters comprise a whole, or a portion of a gene cluster that encodes one or more gene product(s) that are part of a specific biosynthetic pathway for secondary metabolites. In a preferred embodiment of the present invention, the gene clusters encode e.g. multienzyme systems responsible for the synthesis of secondary metabolites. In one embodiment of the present invention, the gene clusters that are desired to be cloned encode the secondary metabolites such as beta-lactams, alkaloids, terpenoids, glycosides, natural phenols, phenazines, biphenyls and dibenzofuranslarge, polyketides, complex glycosides, nonribosomal peptides, and hybrids of the above or hybrids of the afore-mentioned. In a preferred embodiment, the gene clusters intended for being cloned and/or transferred within the meaning of the present invention encode any known or putative cluster of genes for which at least one gene is predicted to encode an activity associated with biosynthesis of secondary metabolites as known in the art.

In this context, beta lactams can be selected from the group consisting of an antibiotic or a lactamase inhibitor. Well known examples of beta lactams are penicillins, cephalosporins, cephamycins, carbapenems, clavams etc.. Alkaloids can for instance be selected from the group consisting of an analgetic, an alkaloid acting on adrenergic system, an antimalarial, etc.. Well known examples of alkaloids are ergot alkaloids, opioids, quinines, reserpine, caffeine, and the like. Glycosides can for instance be selected from the group consisting of an antibiotic and a cardiotonic. Well known examples of glycosides are streptomycine, kanamycine, gentamycine, tobramycine, digitoxine, digoxine. Terpenoids can for instance be selected from the group consisting of anticancer agents, an antimalarial agent and an anticoagulant. Well known examples of terpenoids are for instance taxol, artemisine, coumarines etc. Hybrids of the afore-mentioned chemical groups can for instance be selected from the group of an antibiotic, an antifungal, an anticancer agent, an antigout agent, or an inhibitor of lipases. Further examples can be derived from suitable textbook literature or further publications, such as David J. Newman and Gordon M. Cragg, 2007, Natural Products as Sources of New Drugs over the Last 25 Years, J. Nat. Prod. 2007, 70, 461-477.

The polyketide can for instance be selected from the group consisting of an antibiotic, an immunosuppressant, an anti-cancer agent, an antifungal agent and a cholesterol lowering agent.

The term "non-ribosomal polypeptides" refers to polypeptides that are synthesized using a modular enzyme complex, which functions much like a conveyor belt. Nonribosomal peptides are confined primarily to unicellular organisms, plants and fungi. All of these complexes are laid out in a similar fashion, and they can contain many different modules to perform a diverse set of chemical manipulations on the developing product. In general, these peptides are cyclic (often with highly-complex cyclic structures), although linear nonribosomal peptides are common. Since the system is modular and closely related to the machinery for building fatty acids and polyketides, hybrid compounds are often found. Oxazoles, thiazoles and their reduced counterparts often indicate that the compound was synthesized in this fashion. Other examples of non-ribosomal polypeptides include vancomycin, thiostrepton, ramoplanin, teicoplanin, gramicidin, and bacitracin.

The DNA fragment to be cloned (and finally being present in the BAC-vector) has a size as defined herein, e.g of from suitably at least about 5 kb, suitably to about 500 kb, preferably of from about 10 kb to about 300 kb. By applying the method(s) according to the present invention, such large DNA fragments can be cloned in their entirety, without the need for a previous preparation and screening of BAC- or Cosmid-libraries.
The BAC-vector comprising the elements \((a_1)\) to \((a_5)\) can be transferred to the donor organism by applying any suitable method that is known to a person skilled in the art, e.g. via conjugation. By doing so, in a first step the vector to be transferred is for instance introduced via electroporation into an electrocompetent cell such as \(E.\) coli (e.g. \(E.\) coli strain ET1.2567) that additionally contains a conjugation helper plasmid. Afterwards, conjugation procedure is carried out under appropriate conditions that are known to a person skilled in the art. A suitable protocol is for instance described in Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN-7084-0623-8).

As a donor organism, for instance \(S.\) clavuligerus is used.

After having transferred the BAC-vector into the donor organism, a selection step for the donor organism that contains the BAC-vector incorporated into its genome (this means the exoconjugant) can be carried out. Such a selection step can be carried out by applying any suitable method that is known to a person skilled in the art. Some of said methods are described elsewhere herein. In a preferred embodiment, the selection is carried out based on the presence of an antibiotic resistance marker in the transferred DNA (e.g. apramycin resistance).

In a next step, the genomic DNA from the donor organism is isolated. This isolation step can be carried out by any suitable method that is known to a person skilled in the art. One possible protocol is for instance described in Keiser T., Bibb M.J., Chater K.F., Hopwood D., 2000. Practical Streptomyces genetics. Norwich, John Innes Foundation.

The isolated genomic DNA can then be cleaved, purified e.g. by using the phenol/chloroform extraction and ethanol precipitation protocol from Sambrook, and Russell, 2000. Molecular Cloning: A Laboratory Manual, ISBN-978-087969577-4, and self-ligated in a cyclisation reaction. After ligation, a further DNA purification step can be carried out. In general, the genomic region of the gene cluster to be cloned is known, including restriction enzyme sites. Therefore, the restriction enzymes that are used for carrying out the cleavage reaction are chosen such that the resulting cleaved DNA fragments preferably comprise the whole gene cluster to be cloned. In a preferred embodiment, one restriction site is located at the border of the targeted gene dusted, opposite to the BAC integration site (homologous cluster) and the second site is located in the BAC-vector, adjacent to the target homologous fragment. More precisely the BAC contained restriction site is located at the border of the homologous fragment on the side pointing towards the targeted gene cluster. Therefore, an improved BAC vector can be provided wherein majority of the BAC backbone restriction sites are removed and a large multiple cloning site is integrated. The cyclized rescued DNA fragments represent, or are part of, respectively, a BAC-vector, further comprising the rescued DNA fragment (e.g. the gene cluster or parts thereof) that was located adjacent to the integration site in the donor organism’s genome.

In a next step, the obtained genomic DNA which is now present in a BAC-vector derived from the BAC-vector used in step (a) is transferred into a prokaryotic organism. Said prokaryotic organism the genomic DNA from step (b) or the BAC-vector derived from the BAC-vector used in step (a), respectively, is transferred to can be any suitable prokaryotic organism that is known to a person skilled in the art. In a preferred embodiment, said prokaryotic organism is an Escherichia (\(E.\)) coli strain, preferably an electrocompetent \(E.\) coli strain such as DH5\(\alpha\), and the transfer of DNA is carried out by electroporation.

After the transfer of the genomic DNA that is comprised in the BAC-vector, a selection step is carried out for a prokaryotic organism that contains said transferred BAC-vector. This selection is for instance based on an antibiotic resistance, e.g. apramycin resistance.

Optionally, the BAC-vector can then be isolated form the prokaryotic organism by using a suitable protocol, e.g. by using the PhasePrep BAC DNA Kit (Sigma, USA) according to the manufacturer’s recommendations. As already
described elsewhere herein this step is optional. For instance, a helper plasmid allowing conjugal transfer can be directly provided to the DH5 strain harbouring the BAC-vector with the cloned fragment. In this case this strain is directly used for transfer to host organism. Alternatively, the BAC-vector can be rescued already into a ET1 2567 pUZ8002 strain. Another alternative is a so called three-patental conjugal transfer.

Finally, the optionally isolated BAC-vector is introduced into the host cell, e.g. via conjugation as already described elsewhere herein, followed by an attP/B site/integrase mediated integration of the BAC-vector in the genome of the host cell.

If the plasmid rescue method (ii) is carried out, in step (i) the optionally isolated BAC-vector has to be introduced into a host cell that is provided with a functional integrase, e.g. a functional 0C31 integrase. If the plasmid rescue method (i) is carried out, it is not necessary to provide the host cell with an integrase.

However, independent of which method (i) or (ii)) is used, by applying the method(s) according to the present invention the BAC-vector derived from the BAC-vector of step (a) that comprises the genomic DNA of the donor organism, which genomic DNA, in turn, comprises the DNA fragment to be cloned, does not have to be modified prior to its introduction into the host cell and, preferably, into the host cell's genome. Such a BAC-vector that does not have to be modified is for instance the BAC-vector of step (c), (d), (e), and/or (f). In a preferred embodiment, the BAC-vector from step (c) is not modified during steps (c) to (f). In a further preferred embodiment, the BAC-vector from step (c) is not modified during steps (c) to (f) prior to its introduction into the host cell and, preferably, into the host cell's genome. This is an advantage over prior art protocols, as in the methods described in prior art modification step(s) was (were) necessary, e.g. for ensuring proper integration in the hosts cell's genome. Within the meaning of the present application, the term "modification step" or "modification" denotes any (genetic) manipulation e.g. carried out by a human that is carried out on the BAC-vector that results in an amended BAC-vector, amended e.g. in terms of the nucleic acid sequence of the vector. A modification step can for instance be a cloning step. Such a cloning step e.g. results in the deletion or addition of functional elements such as attP site(s), origin of replication(s), integrase encoding sequence(s), attP-site integrase cassette(s), or in the addition or deletion of restriction enzyme sites. In a preferred embodiment, no attP-site, attP-site integrase cassette, and/or integrase cassette is introduced into said BAC-vector. By avoiding said manipulation step(s) (or by avoiding further cloning step(s), respectively), time-consuming and laborious working steps can be avoided. This avoidance is all the more advantageous as manipulation of large DNA fragments (e.g. fragments having a length of more than 30 kb) is difficult and complicated as standard protocol methods do not apply.

The host cell can be any suitable cell that is known to a person skilled in the art. The host cell can be any microorganism which has the ability to accept foreign DNA via conjugal transfer. A preferred host cell additionally has a C31 specific attB site in the genome, and/or supports replication of the BAC replicon. As exemplified below, both *E. coli* and *Streptomyces*, taxonomically very distant organisms, can be used as hosts. Therefore, the host organism may be selected from any microorganism which is convenient for cultivation and preferably has metabolic properties supporting the synthesis of the cloned cluster encoded secondary metabolites. Such organisms may be selected from, but are not limited to, the group consisting of the genera Streptomyces, Saccharopolyspora, Nocardia, Pseudonocardia, Amycolatopsis, Actinobacterium, Pseudomonas, Gluconobacter, Xantomonas, Burkholderia, E. coli, Bacillus, Mixococcus, Lactobacillus, Lactococcus, Bifidobacterium, Comamonas, Corynebacterium, Brevibacterium, Rhodococcus, Azotobacter, Citrobacter, Enterobacter, Clostridium, Klebsiella, Salmonella, Saccharomyces, Zygossachcharomycyes, Pichia, Klyveromyces, Candida, Hansenula, Dunahella, Debaryomyces, Mucor, Torulopsis, Serratia, Rhizobium, Zymomonas, Propionibacterium, Acetobacter, Arthrobacter, Ralstonia, Sporangium, Chondromyces, Gluconobacter, Propionibacterium, Aspergillus, Neurospora, Chrysosporium, Thielavia, Neurospora, Aureobasidium, Fibbasidium, Piromyces, Cryptococcus, Acremonium, Tolypocladium, Scytalidium, Schizphyllum, Sporotrichum, Penicillium, Mucor, Fusarium and Trichoderma; preferably the host cell is selected from the group
consisting of Streptomyces, Saccharopolyspora, Nocardia, Pseudonocardia, Amycolatopsis, Pseudomonas, Xantomonas, Burkholderia, Bacillus, Mixococcus, Lactobacillus, Lactococcus, Brevibacterium, Bifidobacterium, Comamonas, Corynebacterium, Rhodococcus, Saccharomyces, Pichia, Kluveromyces, Candida, Serratia, Rhizobium, Sporangium, Chondromyces, Gluconobacter, Aspergillus, Neurospora, Acremonium, Penicillium, Mucor and Escherichia coli;

preferably, the host cell belongs to the genus Streptomyces and, more preferably, is selected from the group consisting of Streptomyces clavuligerus, Streptomyces avermitilis, Streptomyces ambofaciens, Streptomyces fradiae, Streptomyces griseus, Streptomyces hygroscopicus, Streptomyces lavendulae, Streptomyces lividans, Streptomyces natalensis, Streptomyces platensis, Streptomyces rimosus, Streptomyces venezuelae, Pseudonocardia autotrophica, Amycolatopsis mediterranei, Amycolatopsis orientalis, Saccharopolyspora erithrea, Saccharopolyspora hirsute, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas syringae, Penicillium citrinum, Penicillium chrysogenum, Xantomonas oryzae, Burkholderia cenocepaica, Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Mixococcus xanthus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus brevis, Lactococcus lactis, Brevibacterium casei, Brevibacterium oxydans, Bifidobacterium bifidum, Bifidobacterium longum, Comamonas sp., Corynebacterium glutamicum, Rhodococcus sp., Aspergillus nidulans, Aspergillus niger, Aspergillus terreus, Saccaromyces cerevisiae, Pichia pastoris, Kluveromyces lactis, Candida albicans, Candida antarctica, Serratia marescens, Rhizobium trifolii, Rhisobium radiobacter, Sporangiumellulosum, Chondromyces croatus, Gluconobacter oxydans, Neurospora crassa, Acremonium chrysogenum and Mucor mucor;

and most preferably, the host cell is selected from the group consisting of S. clavuligerus, S. lividans, S. avermitilis, and E. coli.

The present invention also refers to a BAC-vector comprising (1) an, preferably one, origin of replication, (2) an origin of transfer, (3) a site-specific recombination system containing an attP site however no functional integrase, and (4) a selection marker; as well as to a vector comprising (1) an, preferably one, origin of replication, (2) an origin of transfer, (3) a site-specific recombination system containing a functional integrase and an attP site, (4) a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism, and (5) a selection marker.

In a preferred embodiment of the present invention, the above vectors are BAC-vectors suitable for cloning and/or transfer of a DNA fragment, preferably as described herein. As described above, for this purpose, the respective vector further comprises a DNA sequence that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of a donor organism. In a further preferred embodiment, the vectors are used in methods (I) and (II) as described elsewhere herein, with the vector having no functional integrase preferably being used in method (II) and the vector containing an integrase and an attP site preferably being used in method (I). With regard to the terms "DNA fragment", "homologous recombination", "BAC-vector", "donor organism", "origin of replication", "site-specific recombination system containing an attP site however no functional integrase", "selection marker" and "DNA sequence that allows for homologous recombination", reference is made to the disclosure elsewhere herein, as well as with the use of said vector.

Further as described above, after having carried out homologous recombination with the genome of a donor organism, the vector further comprises a genomic DNA of the donor organism. This genomic DNA comprises a DNA fragment that is intended to be cloned or transferred, preferably said DNA fragment is as defined elsewhere herein.

Further, the present invention refers to the use of the above vectors for cloning and/or transferring of a DNA fragment as described elsewhere herein.

Further, the present invention refers to a donor organism, preferably to a donor organism as described elsewhere herein, comprising a BAC-vector as defined in item (1) (a1) to (a5) or as defined in any one of items (24) or (25), or
(27) to (32) and throughout the specification, and wherein said donor organism does not contain a functional attB site. In a preferred embodiment, said BAC-vector comprises a 0C31 integrase. The donor organism comprising the above BAC-vector (i.e. the BAC-vector comprising a functional integrase and an attP site) is preferably used in method (I) as described herein.

Additionally, the present invention refers to a donor organism, preferably to a donor organism as described elsewhere herein, comprising a BAC-vector as defined in item (3) (a1) to (a5) or as defined in any one of items (26) to (32), and throughout the specification. The donor organism comprising the above BAC-vector, i.e. the BAC-vector comprising an attP site however no functional integrase, is preferably used in method (II) as described herein.

In a preferred embodiment, the BAC-vectors of the respective donor-organism are shuttle-BAC-vectors, preferably E. coli - Actinomycetes conjugative vectors.

In a further preferred embodiment, the origin of replication of the respective BAC-vector is ori S, and/or the origin of transfer is ori T.

In a further preferred embodiment, the site-specific recombination system of the respective BAC-vector is a 0C31 attP-int recombination system.

In a further preferred embodiment, the respective BAC-vectors comprise a prokaryotic F factor partitioning system.

The present invention also refers to an expression system, comprising

(1) a host cell, and

(2) a BAC-vector, wherein said BAC-vector comprises

i) an, preferably one, origin of replication;

ii) an origin of transfer;

iii) a site-specific recombination system containing an attP site and a functional integrase;

iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more
gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and

v) a selection marker.

With regard to the terms "expression system", "host cell", "functional integrase", "BAC-vector", "origin of replication", "origin of transfer", "site-specific recombination system", "attP site"; "selection marker", "DNA fragment", "gene cluster", and "biosynthetic pathway for secondary metabolites", reference is made to the description elsewhere herein. Further, in one embodiment, the features (i, ii, iii) and v) of the BAC-vector correspond to features (a1), (a2), (a3) and (a5) of the BAC-vector of item (1), and/or to features (1), (2), (3) and (4) of the vector of item (24). In a preferred embodiment, the expression system is used within method (I) as described elsewhere herein.

The present invention further refers to an expression system, comprising

(1) a host cell, wherein said host cell is provided with a functional integrase, and

(2) a BAC-vector, wherein said BAC-vector comprises

i) an, preferably one, origin of replication;

ii) an origin of transfer;

iii) a site-specific recombination system containing an attP site however no functional integrase;

iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more
gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and

v) a selection marker.
With regard to the terms "expression system", "host cell", "functional integrase", "BAC-vector", "origin of replication", "origin of transfer", "site-specific recombination system", "attP site"; "selection marker", "DNA fragment", "gene cluster", and "biosynthetic pathway for secondary metabolites", reference is made to the description elsewhere herein. Further, in one embodiment, the features i), ii), iii) and v) of the BAC-vector correspond to features (a1), (a2), (a3) and (a5) of the BAC-vector of item (3), and/or to features (1), (2), (3) and (4) of the vector of item (26). In a preferred embodiment, the functional integrase the host cell is provided with is a OC31 integrase. In a further preferred embodiment, the expression system is used within method (II) as described elsewhere herein.

Further, the present invention refers to the use of the expression system for cloning and/or transfer of a DNA fragment as described herein, and/or for expression of said DNA fragment. Preferably, the DNA fragment is a DNA fragment as described elsewhere herein.

Further, the present invention refers to a vector, preferably a BAC-vector, comprising a sequence according to SEQ ID NO: 3 (SEQ ID NO: 3 is depicted in Fig. 3 b)), and a BAC-vector comprising SEQ ID NO: 3 is for instance the vector pBeloBAC1 1+pSET1 52, depicted in Fig. 3 a)), or to SEQ ID NO: 53 (depicted in Fig. 7 b)). SEQ ID NO: 53 represents an improved version of the BAC-vector comprising SEQ ID NO: 3 in that the BAC-vector comprising SEQ ID NO: 53 has deleted restriction sites in the backbone. A BAC-vector that comprises SEQ ID NO: 53 is for instance the vector pBAC_LEK, depicted in Fig. 7 a).

In a preferred embodiment, said vectors additionally comprise a DNA sequence that allows for homologous recombination with a corresponding substantially (or even completely) homologous DNA sequence being present in the genomic DNA of a donor organism. Further preferred, the vectors comprise a DNA fragment as described elsewhere herein. In a further preferred embodiment, the vectors are used for cloning and/or transfer of a DNA fragment, and/or for expression of said DNA fragment.

Therefore, the present invention also refers to the use of the above vectors for cloning and/or transfer of a DNA fragment, and/or for expression of said DNA fragment. In a preferred embodiment, the vectors are used in method (I) as described herein.

Description of the figures

Fig. 1: BAC-vector pUJS.
Figure 1 shows the BAC-vector pUJS.

Fig. 2: Schematic view of gene cluster cloning.
Figure 2 shows a schematic view of gene cluster cloning. The part of the genome sequence represented is GeneBank PRJNA42475: 142996-21 101 7.

Fig. 3: pBeloBAC1 1+pSET1 52
Figure 3 a) shows the vector pBeloBAC1 1+pSET1 52.
Figure 3 b) shows the various features and the nucleotide sequence (SEQ ID NO: 3) of the BAC-vector pBeloBAC1 1+pSET1 52.

Fig. 4: pBeloBAC1 1+pSET1 52-C31
Figure 4 a) shows the vector pBeloBAC1 1+pSET1 52-C31.
Figure 4 b) shows the various features and the nucleotide sequence of the BAC-vector pBeloBAC1 1+pSET1 52-C31 (SEQ ID NO: 6).
Fig. 5 : pUJS-C31
Figure 5 shows the BAC-vector pUJS-C31.

Fig. 6 : pUJS+Clavulanic cluster (BamHI)
Figure 6 shows the BAC-vector pUJS+Clavulanic cluster (BamHI).

Fig. 7 : pBAC_Lek
Figure 7 a) shows the BAC-vector pBAC_Lek
Figure 7 b) shows the various features and the nucleotide sequence of the BAC-vector pBAC_Lek (SEQ ID NO: 53). pBAC_Lek is a vector having deleted restriction sites in the backbone. It has a multiple cloning site containing more than 50 unique restriction sites. All duplicate deletion sites have been deleted from the rest of the BAC-vector backbone. The following unique restriction sites are present in the multiple cloning site: BgIII, BstBII, AlIII, Clal, EcoRV, HindIII, KpnI, Acc65I, Ndel, Sali, Bsu36I, Saul, Stul, BstBI, XbaI, Muni, Mfel, AclI, PvuII, BspEI, Hpal, NruI, SexAI, Mscl, SnaBI, AplI, Ascl, BssHII, PvuII, AsISI, AvrII, BamHI, BfrBI, Nsai, Ppol, SpfI, Nhel, BmtI, BstZ1I, FseI, EcoRI, NotII, PacI, Asel, Pmel, Dral, SacII, SblI, PstI, Seal and SrfI. Further, pBAC_Lek comprises one origin of replication, an origin of transfer, a site-specific recombination system containing an integrase and an attP site, and a selection marker.

Examples

General Molecular biology methods

The PCR reactions were carried out with PCR Extender System (5PRIME) and the buffer provided by the manufacturer (10x tuning buffer with magnesium) in the presence of 200 μM dNTP, 4% DMSO, 2 μM of each primer, approximately 50 ng of template DNA (or 1μl when colony PCR templates were used) and 1 unit of enzyme in a final volume of 25 μl for 30 cycles. The thermal profile started with a denaturation step at 94°C for 10 min. The thermal cycle for the following 30 cycles was 94°C for 45 sec (denaturation step), 45 sec of annealing step with temperatures from 54°C to 64°C (depending on the primer pair used), and 72°C for 150 sec (extension step). The final elongation step was carried out at 72°C for 7 min.

Cleavage with restriction nucleases, T4 DNA ligase reactions, DNA isolation and purification methods were performed according to the recommendations of the manufacturer. General molecular methods are well known in the art but can be reviewed in Sambrook, and Russell, 2000. Molecular Cloning: A Laboratory Manual, ISBN-978-087969577-4.

Example 1: construction of pUJS BAC vector

The pUJS BAC vector was assembled by PCR amplification of a fragment containing 0C31 integrase and attP site, aac(3)/IV gene conferring Apramycin resistance, traJ gene and ori T, facilitating conjugal transfer of plasmids, was amplified from pSET152 (Kieser et al., 2000, Practical Streptomyces genetics, A laboratory Manual. ISBN0-784-0623-8) in a PCR reaction using oligonucleotide primers

CGGCCCGGATGACATCCTAGGTAGGATTATTCAAAAG GATCTTCACCTAG ATCC (SEQ ID NO: 1) and

GGCCCTCGAGGAGCTCGTCGAC AATTCCCAATGTCAGCAGTTCC (SEQ ID NO: 2),
incorporating NsiI, AvrII, XhoI, and BamHI, ApaI, XhoI restriction sites (underlined) respectively. The obtained
fragment was cleaved with XhoI restriction endonuclease and ligated to the large fragment obtained with the
restriction of pBeloBAC1 1 with Sail. The resulting vector
pBeloBACI 1_pSET1 52 (Figure 3 a) and b); SEQ ID NO: 3)
was then cleaved with ApaI and BamHI in order to insert a fragment obtained by cleaving pGEM/homolog with the
same fore mentioned restriction endonuclease. The above fragment is homologous to the target integration site
(genome sequence GenBank PRJNA42475: 174376-174802) for the plasmid rescue cloning and was obtained by
amplification by PCR from the genome of S. clavuligerus (oligonucleotide primers:
CGGGTGTCGACAGTGTTGACGACGGCTATCTCAA
(SEQ ID NO: 4) and
GGCCGGATCCCTCAGGATGGTAGTGATCGATCGGATGATG
(SEQ ID NO: 5))
and cloning into the pGEM-T easy PCR cloning vector (Promega) yielding plasmids pGEM/homolog. The sequence
analysis of the cloned PCR fragment confirmed its respective sequence as expected from the primer design
procedure. The final vector was designated pUJS and its features are shown in Figure 1.

A derivative of the pUJS vector, pUJS-C31 (Fig. 5), was prepared by removing the 0C31 integrase gene via
restriction with SfuI and HindIII, blunt-ending of the cleaved large fragment and cyclisation of the fragment using T4
ligation reaction. Similarly the 0C31 integrase gene was removed also from pBeloBACI 1_pSET1 52 yielding
pBeloBACI 1_pSET1 52-C31 (Figure 4; SEQ ID NO: 6).

The produced vector does not code functional OC31 integrase, however the attP site, recognized by the integrase is
conserved.

Example 2: Isolation of S. clavuligerus ATCC27064 OC31 affS deletion mutant

i) Gene deletion construct

The plasmid pKONC was designed as a suicide plasmid for Streptomyces. Only E. coli replicative element is included
ISBN0-7804-0623-8), therefore upon transformation and exposure to selection pressure only those cells of S.
clavuligerus form colonies that have the plasmid integrated into the chromosome. Two selection markers are included
into the plasmid: aac(3)IV gene conferring apramycin resistance, originating from pSET152, a well known plasmid for
and codA counterselection marker gene originating from pMG302M, producing a toxic 5-fluoro-uracil in presence of
5-fluoro-uracil (Dubeau, et al., 2009).

codA gene was amplified using PCR amplification of pMG302M plasmid DNA using oligonucleotide primers
GGGCCATATGGTTGACATCTTTTGCGGATGCTC
(SEQ ID NO: 7) and
GGGCCATATGGTTGACATACCTTTTGCGGATGCTC
(SEQ ID NO: 8).
The PCR-amplified product was fragment was cloned into the pGEM-T easy PCR cloning vector (Promega) yielding
plasmid pGEM/codA. The sequence analysis of the cloned PCR fragment confirmed its respective sequence as
expected from the primer design procedure.
aac(3) IV gene was amplified using PCR amplification of pSET152 plasmid DNA (Kieser et al., 2000, Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) using oligonucleotide primers

CGGCTCTAGAGATCTTACCACTAGATCCCTTTGTTTC (SEQ ID NO: 9) and
CCGGGAATTCCTCCGATCCGCTCCACGTG (SEQ ID NO: 10).

The PCR-amplified product was cloned into the pGEM-T easy PCR cloning vector (Promega) yielding plasmids pGEM/Apr2. The sequence analysis of the cloned PCR fragment confirmed its respective sequence as expected from the primer design procedure. The plasmid pGEM/CodA (as described above) was cleaved using restriction endonucleases Ndel and Xbal (both Promega), the mixture was resolved on the agarose gel electrophoresis gel. The resulting 1368 bp fragment was purified from the agarose gel. In parallel the plasmid pGEM/Apr2 was cleaved with the restriction endonucleases Ndel and Xbal (Both Promega) so that 3987 bp was isolated from the band cut out of an agarose electrophoresis band. The fragments were purified of the remaining agarose and assembled in a DNA ligation reaction using T4 DNA Ligase (Promega) to yield pGEM/codA_Apr2 with overall length of 5357 bp.

Plasmid pSET151 (Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) was then cleaved using restriction endonuclease EcoRI and Ndel (both Promega) under conditions of partial restriction. 5897 bp fragment containing the larger part of the plasmid was purified of the remaining agarose. In parallel, the plasmid pGEM/codA_Apr2 was cleaved with the restriction endonucleases Ndel and EcoRI (Both Promega) so that 2373 bp fragment containing aac(3) IV and codA genes was isolated from the band cut out of an agarose electrophoresis band. The fragments were assembled in a DNA ligation reaction using T4 DNA Ligase (Promega), yielding pSET151/codA_Apr2 with overall length of 8270 bp.

Finally, the plasmid pSET151/codA_Apr2 was cleaved using restriction endonuclease Dral (Promega) and 5428 bp fragment containing the larger part of the plasmid was purified and selfligated in a DNA ligation reaction using T4 DNA Ligase (Promega) yielding pKONC with overall length of 5428 bp which differs from pSET151/codA_Apr2 in loss of fragment containing genes xylE, tsr and bla. The resulting clones were verified using restriction analysis with several distinct restriction endonucleases.

ii) Gene deletion construct with 0C31_attB flanking fragments:

The DNA fragments, flanking the attB site from either side were amplified by PCR from S. clavuligerus genomic DNA using oligonucleotide primers

GCGCGAATTCCCAGGATGGACTTCCGGGTCA (SEQ ID NO: 11) and
GCGCGATCCCGGATGGAAGACCTCCT (SEQ ID NO: 12)

for the amplification of the 5’ flanking region of attB, and oligonucleotide primers

CGGCGGATCCAGCCCTCCAGCTTACGTGGTA (SEQ ID NO: 13), and
GCCGAAGCTTACCTGATGAGCGCCTT (SEQ ID NO: 14)

for the amplification of the 3’ flanking region of the attB.

The fragments were cloned into the pGEM-T easy PCR cloning vector (Promega) yielding plasmids pGEM/attB_L1 containing 5’ flanking fragment and pGEM/attB_D1 containing 3’ flanking fragment. The sequence analysis of the cloned PCR fragments confirmed its respective sequence as expected from the primer design and genomic sequence. The plasmid pKONC (as described above) was cleaved using restriction endonucleases EcoRI and Hind III (both Promega) and the 5377 bp fragment was purified from the agarose gel. In parallel the plasmid pGEM/attB_L1 was cleaved with the restriction endonucleases EcoRI and BamHI (Both Promega) so that 976 bp 5’ flanking fragment was isolated. The 1079 bp 3’ fragment was excised from pGEM/attB_D1 using BamHI and Hind III and
purified. The fragments were assembled in a single step DNA ligation reaction using T4 DNA Ligase (Promega) yielding the final construct designated pKONC/attB with overall length of 7454 bp.

iii) Selection of the ΦC31 attB deletion strain.

Plasmid construct pKONC/attB was introduced by transformation into electrocompetent E. coli strain ET1-2567 containing the conjugation helper plasmid pUZ8002 (Paget et al., 1999 J. Bacteriol. 181: 204-211). Conjugation procedure was done as described in Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) using S. clavuligerus ATCC 27064 spores. KKA-2L agar plates were used for selection of exoconjugants and apramycin (Sigma) was used for selection at 60 µg/ml. Transformation with vector pKONC/attB yielded 12 exoconjugants resistant to apramycin. As the plasmid pKONC/attB is unable to replicate itself in S. clavuligerus, the exoconjugants must have had the said plasmid integrated into the genome. Only tops of exoconjugant colonies were transferred in a dense spread to KKA-2L medium supplemented with 60 µg/ml apramycin (KKA-2L Apr) and grown at 25°C for 10 days. Exoconjugants were tested by a PCR method, described later in detail, and were shown that they all contain a single crossover genotype as expected.

Selection for stable secondary crossover recombinant strains of S. clavuligerus with disrupted attB site was achieved by counterselection to codA (Cytosine-deaminase) marker gene with fluoro-cytosine (Sigma) and therefore loss of all genetic markers together with the pKONC/attB plasmid backbone. The approach is known by skilled persons as scarless gene deletion (Dubeau et al., 2009).

**Table 1.:** The composition of the KKA-2L medium and the mineral solution used within.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn dextrose (ROUETTE)</td>
<td>10 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1 g</td>
</tr>
<tr>
<td>MgSO₄ × 7H₂O</td>
<td>1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ × 7H₂O</td>
<td>2 g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>4 g</td>
</tr>
<tr>
<td>Mineral solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>Bacto agar (DIFCO BP)</td>
<td>20 g</td>
</tr>
<tr>
<td>Tap water</td>
<td>To a final volume of 1 liter</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M HCl</td>
<td>10 ml</td>
</tr>
<tr>
<td>CaCl₂ x 2H₂O</td>
<td>25 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>12 g</td>
</tr>
<tr>
<td>MgCl₂ x 6H₂O</td>
<td>66 g</td>
</tr>
<tr>
<td>KCl</td>
<td>100 g</td>
</tr>
<tr>
<td>FeCl₃ x 6H₂O</td>
<td>5 g</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.7 g</td>
</tr>
<tr>
<td>CuCl₂ x 2H₂O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>MnSO₄ x 2H₂O</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Ultra pure water</td>
<td>To a final volume of 1 liter</td>
</tr>
</tbody>
</table>

The pH is naturally ~ 7.0, there is no pH adjustment prior to sterilization. Sterilization is performed at 121±2°C, 220±10 kPa for 20 minutes.

Spores of S. streptomyces exoconjugants with vector pKONC/attB, grown at 25°C on KKA-2L medium supplemented with 60 µg/ml apramycin were inoculated into liquid SNK-t medium with apramycin (60 µg/ml) and grown in deep-well microtiter plates at 28°C and 260 rpm to produce dense mycelium culture. After 24h, this culture was subcultivated in parallel wells of fresh deep-well microtiter plates, one containing SNK-t medium supplemented with 60 µg/l apramycin and the other containing SNK-t medium supplemented with 100 µg/l 5-fluoro-cytosine (FC) medium and grown at
25°C and 260 rpm until growth appeared in the wells containing 5-fluoro-cytosine. The cultures were subcultivated repeatedly in parallel wells, as soon as growth appeared in the SNK-t FC containing wells in a manner described above (the 0.1 % inoculum used, was always taken from the preceding generation in SNK-t FC wells).

Table 2.: The composition of the SNK-t medium.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Soytone (DIFCO BD)</td>
<td>20 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glycerol (Sigma)</td>
<td>24 g</td>
</tr>
<tr>
<td>soya oil (GEA)</td>
<td>5 g</td>
</tr>
<tr>
<td>Ultra-pure water</td>
<td>To a final volume of 500 ml</td>
</tr>
</tbody>
</table>

The procedure was repeated for as little as three to as many as ten cycles of selection, until growth was no longer appearing in the wells with SNK-t supplemented with apramycin. The cultures were then plated onto ISP-2 medium containing 100 µg/ml 5-fluoro-cytosine in appropriate dilutions yielding approximately 100 colonies per plate. After 5-10 days, the colonies were spread in parallel onto KKA-2L and KKA-2L Apr agar plates. A large majority of the transferred strains showed growth only on KKA-2L but not on KKA-2L Apr agar plates indicating loss of genetic markers from the genome and thereby successful secondary recombination. Approximately 30 independently obtained secondary recombinants were isolated.

iv) Conformation of deletion of the 4C31 attB by genetic characterization.

PCR was used for conformation of correct integration and homologous recombination events during the selection pressure cycles. A set of conformation oligonucleotide primers were designed which distinguish between wild type, single crossover situation and the final double recombination situation in the locus of the attB site in the S. clavuligerus genome.

DNA templates were prepared in with a “colony PCR” approach. Specifically, the spores of the tested strains were cultivated in SNK-t medium at 25 °C and 260 rpm for 48 h, followed by separation of the mycelium by filtration, and partial permeabilization of the mycelium using resuspension in TE buffer (Sambrook, and Russell, 2000. Molecular Cloning: A Laboratory Manual, ISBN-978-087969577-4), supplemented with 2.5 µg/ml RNase (Roche, 500 µg/ml). The mycelium as filtered off and the filtrated was used as a DNA template for the PCR reactions with the oligonucleotide primer pair

ACGATGATGCTACCCACCAGTAATAAGTTG  (SEQ ID NO: 15) and
GACATAGCGAGTCCGTGAAGTCT  (SEQ ID NO: 16).

Expected amplicon lengths are given in table 3.

Table 3.: The expected lengths of the conformation PCR products

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Single cross-ower</th>
<th>Double cross-ower</th>
</tr>
</thead>
<tbody>
<tr>
<td>expected length</td>
<td>1399 bp</td>
<td>994 bp, 1399 bp</td>
<td>994 bp</td>
</tr>
</tbody>
</table>
5 strains originating from *S. clavuligerus* ATCC 27064 had the expected PCR product profile. These strains were additionally tested for absence of genetic marker cytosine deaminase and marker for apramycin resistance using PCR and were all found negative. Other strains have reverted to the wild type genotype and were therefore discarded. From the 5 isolated strains, one (S. *clavuligerus* ATCC 27064 -latB) was selected for further studies.

**Example 3: Characterization of integration frequencies in S. clavuligerus OC31 attS deletion mutant**

Several vectors were prepared in order to study the frequency of integration via homologous recombination v.s. phage integrase driven integration into the genome of *S. clavuligerus* ATCC 27064 w.t. and *S. clavuligerus* ATCC 2706 AattB. In addition role of putative pseudo-attB sites in *S. clavuligerus* ATCC 27064 was investigated.

pSET1 52 (Kieser et al., 2000, Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) was used as the basis of further modified vectors.

pSET1 52_attP was prepared by modification of the 0C31 attP site located on the pSET1 52 plasmid. Targeted PCR mutagenesis was used using QuickChange site-directed mutagenesis kit (Stratagene, USA) and oligonucleotide pair

\[
\begin{align*}
\text{CCAACCTGGGGTAC} & \quad \text{CTCGAGTTCTCGATTGGGGGCGT} \quad (\text{SEQ ID NO: 17}) \\
\text{AGGCCCCCAACTGAGAGAA} & \quad \text{CTCGAGTTACCCCAAGTGG} \quad (\text{SEQ ID NO: 18}).
\end{align*}
\]

The underlined part shows the Xhol restriction site which was introduced with the site directed mutagenesis procedure, thereby replacing the original sequence CTCTGAG containing the thymidine pair critical for 0C31 integrase function on the site with the CTC-GAG. In this way a vector was designed which is practically homologous to the pSET1 52 however its integration into the chromosome through 0C31 integrase action was disabled.

Into both pSET1 52 and pSET1 52_attP, a fragment homologous to the target site in the genome of *S. clavuligerus* ATCC 27064 was inserted by first cleaving the above vectors and plasmid pGEM/homolog (described in example 1) with EcoRI restriction endonuclease and the assembling the fragments in a ligation reaction to obtain pSET1 52/homolog and pSET1 52_attP/homolog.

In addition, to check the integration frequency of alternative phage integrase, specifically ΦBT1 integrase, a pSET1 52 type vector having the 0C31 integrase replaced by ΦBT1 integrase was constructed. ΦBT1 integrase gene was chemically synthesized based on the sequence deposited by Gregory et al., 2003 with the GeneBank accession number AJ550940 (sequence position 38669-40697). In addition Sphi and BamHI recognition sites were added at 5' and 3' end respectively. The pSET1 52 was cleaved with BamHI and Sphi restriction endonucleases and the large fragment was ligated to BamHI, Sphi cleaved ΦBT1 integrase, ΦBT1 attP site fragment. The resulting plasmid was designated pSET1 52/BT1

Plasmids pSET1 52, pSET1 52_attP, pSET1 52/homolog, pSET1 52_attP/homolog, pAET/BT1 were introduced by transformation into electrocompetent *E. coli* strain ET1 2567 containing the conjugation helper plasmid pUZ8002 (Paget et al., 1999 J. Bacteriol. 181: 204-211). Conjugation procedure was done as described in Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) using *S. clavuligerus* ATCC 27064 w.t. and *S. clavuligerus* ATCC 27064 AttB spores. KKA-2L agar plates were used for selection of exconjugants and apramycin (Sigma) was used for selection at 60 µg/ml. The experiments were conducted with utmost care that the conjugation conditions were equal for all tested plasmids and were repeated four times independently.
Table 4.: Frequency of integration, based on the number of exoconjugant colonies after four independent experiments of conjugal transfer.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of exoconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pSET1 52</td>
</tr>
<tr>
<td></td>
<td>&gt;100000</td>
</tr>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pSET1 52/BT1</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pSET1 52_attP</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pSET1 52_attP/homolog</td>
</tr>
<tr>
<td></td>
<td>115</td>
</tr>
<tr>
<td>S. clavuligerus AattB</td>
<td>pSET1 52</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>S. clavuligerus AattB</td>
<td>pSET1 52_attP</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>S. clavuligerus AattB</td>
<td>pSET1 52/homolog</td>
</tr>
<tr>
<td></td>
<td>45</td>
</tr>
<tr>
<td>S. lividans TK24</td>
<td>pSET1 52</td>
</tr>
<tr>
<td></td>
<td>&gt;100000</td>
</tr>
<tr>
<td>S. lividans TK24</td>
<td>pSET1 52/BT1</td>
</tr>
<tr>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

In another set of experiments, frequencies of integration into the genome of S. clavuligerus ATCC 27064 w.t. and S. clavuligerus ATCC 27064 AattB for BAC plasmids pBeloBAC1_1_pSET1 52, pBeloBAC1_1_pSET1 52-C31, pUJS and pUJS-C31 were studied based on the number of exoconjugants obtained after the conjugation procedure as described above.

Table 5.: Frequency of integration, based on the number of exoconjugant colonies after four independent experiments of conjugal transfer.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of exoconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pBeloBAC1_1_pSET1 52</td>
</tr>
<tr>
<td></td>
<td>350</td>
</tr>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pBeloBAC1_1_pSET1 52-C31</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>S. clavuligerus w.t.</td>
<td>pUJS-C31</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>S. clavuligerus AattB</td>
<td>pBeloBAC1_1_pSET1 52</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>S. clavuligerus AattB</td>
<td>pUJS</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

The results in Table 4. show that:

i) The frequency of integration into the genome of S. clavuligerus w.t., based on the transformation efficiency with pSET1 52/BT1 (Φ8T1 integrase) are drastically lower than efficiency with pSET1 52 (OC31 integrase). This result shows that the Φ8T1 integrase is not very suitable for the BAC based targeted cloning when considering that frequencies of transformation with conjugal transfer are lower for larger, low copy-number vectors (as shown with the OC31 integrase BAC based vectors). A similar result was obtained when S. lividans TK24 was used as a host. This approach was abandoned as it would be difficult to introduce the BAC vectors with large inserts into host organisms especially into S. clavuligerus.

ii) The frequency of integration of pSET1 52_attP into S. clavuligerus w.t. is 0. This confirmed that the vectors used for the study do not have nonspecific means of integration into the S. clavuligerus genome, which would be theoretically possible by means of unknown integration mechanism, some sort of replicative mechanism or homologous recombination with unexpected homology in the genome.

iii) The frequency of integration pSET1 52_attP/homolog is reasonable although a 1000 fold lower than with pSET1 52. Taking into account the previously described result the only way this integration is possible is via homologous recombination. The lower efficiency of integration with homologous recombination than via the
Φ C31 integrase is expected. It is surprising that the frequency of integration with homologous recombination is higher than the frequency of integration via Φ8 T1 integrase - result described under i). This is in contrast with the prior knowledge (Gregory et al. 2003).

iv) The frequency of integration into the genome of S. clavuligerus AattB using pSET1 52 is drastically lower than that of S. clavuligerus w.t. is the host strain. The few detected transformants are likely obtained by integration of pSET1 52 into the "pseudo-attB" sites. This result also suggests that the frequency of integration into the "pseudo-attB" sites via the Φ C31 integrase action is negligible compared to integration into the prime attB site and even compared to integration via homologous recombination - result described in iii).

v) The frequency of integration of pSET1 52/homolog into the genome of S. clavuligerus AattB is approximately half of that obtained by transformation of S. clavuligerus w.t. which is likely due to small differences in spore concentration in conjugation procedure. Still the frequency is sufficiently high for further work in targeted insertion of BAC based vector into the genome of S. clavuligerus.

The results in Table 5. show that:

i) The frequency of integration into the genome of S. clavuligerus w.t. based on the transformation efficiency with pBeloBACI 1_pSET1 52 (Φ C31 integrase) are significantly lower than efficiency with pSET1 52. This result shows that other property of the BAC skeleton has an effect on transformation efficiency, most probably due efficiency of conjugal transfer. The copy number of BAC vectors in the donor E. coli strain is one while pSET1 52 type vectors are multi-copy plasmids.

ii) The frequency of integration of pBeloBACI 1_pSET1 52-C31 into S. clavuligerus w.t. is 0. This shows that the vectors without C31 integrase function or substantial homologous regions to the host organism genome do not have nonspecific means of integration. The frequency of integration of pUJS-C31 which contains such homology is low, however two transformants were obtained with target specific integration of the BAC vector at the site of plasmid rescue cloning. Molecular analysis of the strains showed that integration of the vector occurred by homologous recombination at the correct targeted site in the genome. One of the strains was designated K81 6 and was used for further work. This result shows possibility to use the BAC complete BAC vector in a w.t. gene cluster donor strain. Upon reintegration or heterologous expression of the cloned fragment, Φ C31 integrase function can be provided in trans (such as on temperature sensitive replicative vector) therefore avoiding necessity of preparation of the AattB gene cluster donor strain. Alternatively, as shown in WO2009/01 7692 Φ C31 integrase can be provided by subsequent molecular modification of the large BAC vector, which is often difficult due to special methods necessary for molecular modification of large fragments of DNA.

iii) The frequency of integration of pUJS into S. clavuligerus AattB is low compared to pSET1 52/homolog, likely due to copy number reason. However 10 transformants have been isolated and all have been confirmed at the molecular level to have correct target specific integration in the genome which occurred due to homologous recombination event. One of the strains was designated K771 and was used for further experiments. A schematic figure of the rescued BAC vector comprising the clavulanic cluster is depicted in Fig. 6 ("BAC vector pUJS+Clavulanic cluster (BamHI)").

Example 4: Plasmid rescue procedure
Strains K81 6 and K771 were used in the plasmid rescue procedure. Construction of the BAC vectors used to obtain the aforementioned strains, specifically the homology fragment to the target site in the genome, was such that integration of the vectors occurred in the gene pcbAB in the cephamycin C gene cluster in S. clavuligerus. The targeted gene cluster for cloning was the neighbouring clavulanic acid cluster. Due to integration of the BAC vectors into the pcbAB gene, a peptide synthase essential for cephamycin C synthesis, the target specific integration could be confirmed both by genotype analysis (PCR) and phenotype analysis (LC-MS; abolishment of cephamycin C accumulation). Figure 2. depicts the complete integration and plasmid rescue procedure for one of the cloned clusters. Experimental procedure is described in detail below.

i) Isolation of genomic DNA: Strains K81 6 and K771 were grown in the SNK-t medium at 25 °C and 260 rpm for 48h to obtain mycelium. The genomic DNA was isolated according to the procedure described in Keiser T., Bibb M.J., Chater K.F., Hopwood D., 2000. Practical Streptomyces genetics. Norwich, John Innes Foundation.

ii) ~ 1μg of Genomic DNA was cleaved under standard conditions using several restriction endonucleases. Restriction with each of the 3 selected endonucleases would yield distinct gene cluster cloned into the BAC vector. BamHI cleavage would afford clavulanic acid biosynthesis gene cluster, Saul an elongated clavulanic acid biosynthesis gene cluster and Hind III a partial cephamycin C gene cluster. (Figure 2.)

iii) The cleaved DNA was purified according to the phenol/chloroform extraction and ethanol precipitation protocol from Sambrook, and Russell, 2000. Molecular Cloning: A Laboratory Manual, ISBN-978-089686577-4. The DNA was then self-ligated in a T4 ligase (Promega) cyclisation reaction. The ligation conditions were: 14°C 18h, 1 Weiss unit of T4 ligase per 10μl of ligation mixture. After ligation, the DNA was purified by phenol/chloroform extraction (as above), ethanol precipitated and dissolved in 5μl of pure water.

iv) The 5μl of purified ligation mixture DNA was mixed with 30μl E. coli DH5α Electromax (Invitrogen, USA) electro competent cells and electroporation transformation procedure was carried out as recommended by the manufacturer. The transformants were selected on LB agar plates supplemented with 25μg/ml Ampicillin. The plates were incubated overnight at 37°C. The numbers of transformants obtained for each strain - restriction enzyme combination are given in table 6.

**Table 6.:** Number of E. coli colonies obtained after plasmid rescue procedure starting from either K771 or K81 6 genomic DNA cleaved with BamHI, HindIII or Saul restriction endonuclease.

<table>
<thead>
<tr>
<th></th>
<th>BamHI</th>
<th>HindIII</th>
<th>Saul</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. clavuligerus K771</td>
<td>85</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>S. clavuligerus K81 6</td>
<td>23</td>
<td>34</td>
<td>15</td>
</tr>
</tbody>
</table>

The BAC DNA was isolated from 5 colonies of each group of E. coli DH5α clones using PhasePrep BAC DNA Kit (Sigma, USA) according to manufacturer’s recommendations. The BAC clones were designated pUJS+Clavulanic cluster (BamHI) (for the BamHI clones originating from K771), pUJS/HindIII (for the HindIII clones originating from K771), pUJS+Clavulanic cluster (Saul) (for the Saul clones originating from K771). The BAC clones without 0C31
integrase function were designated pUJS-C31/BamHI (for the BamHI clones originating from K81 6), pUJS-
C31/HindIII (for the HindIII clones originating from K81 6), pUJS-C31/Saul (for the Saul clones originating from K81 6).

Figure 2: Schematic view of gene cluster cloning. The part of the genome sequence represented is GeneBank
PRJNA42475: 142996-21 to 17.

Example 5: Characterization of cloned fragments

The isolated BAC clones were first analyzed by restriction analysis and after conformation of identical restriction
pattern for each group of clones, one of each was selected for sequencing. The shot-gun sequencing was performed
using Roche FLX Titanium sequencing method. The coverage of the sequencing after assembly with Celera
assembler to the reference was higher than 48 for all positions. The ratio of reads assembled to the GeneBank
PRJNA42475 reference and reads assembled to the pUJS vector backbone sequence corresponds precisely to the
ratio of the length of insert to the length of the pUJS vector backbone. The sequencing result show that the cloned
fragment corresponds to the reference GeneBank PRJNA42475 with 100% identity. The cloned fragment length for
pUJS+Clavulanic cluster (BamHI) and pUJS-C31/BamHI was 29892 bp and matched position between 174379 and
204271 on the GeneBank PRJNA42475 reference. The cloned fragment length for pUJS/HindIII and pUJS-
C31/HindIII was 57433bp and matched position between 116727 and 174160 on the GeneBank PRJNA42475
reference. The cloned fragment length for pUJS+Clavulanic cluster (Saul) and pUJS-C31/Saul was 50427bp and
matched position between 174379 and 224806 on the GeneBank PRJNA42475 reference.

Example 6: Transfer of cloned clusters to S. clavuligerus and S. lividans

Vectors pUJS+Clavulanic cluster (BamHI) and pUJS+Clavulanic cluster (Saul), were introduced by transformation
into electrocomptent E. coli strain ET152567 containing the conjugation helper plasmid pUZ8002 (Paget et al., 1999
J. Bacteriol. 181: 204-21 1). Conjugation procedure was done as described by Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN0-7084-0623-8) using S. clavuligerus ATCC 27064 w.t. and S. lividans TK24 spores. KKA-2L agar plates were used for selection of exoconjugants and apramycin (Sigma) was
used for selection at 60 µg/ml. The experiments were conducted with utmost care that the conjugation conditions
were equal for all tested plasmids. The number of colonies obtained in three independent experiments is shown in
table 7.

Table 7: The number of exoconjugant colonies obtained after conjugal transference of secondary metabolite clusters to
S. clavuligerus and S. lividans.

<table>
<thead>
<tr>
<th></th>
<th>pUJS+Clavulanic cluster (BamHI)</th>
<th>pUJS+Clavulanic cluster (Saul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. clavuligerus ATCC 27064</td>
<td>31, 27, 43</td>
<td>18, 16, 27</td>
</tr>
<tr>
<td>S. lividans TK24</td>
<td>233, 177, 262</td>
<td>132, 105, 165</td>
</tr>
</tbody>
</table>

Table 7: The number of exoconjugant colonies obtained after conjugal transference of secondary metabolite clusters to
S. clavuligerus ATCC 27064 and S. lividans.
The presence of heterologous genes in S. lividans exoconjugants was confirmed by colony PCR amplification of a number of smaller fragments from the cloned secondary metabolite gene cluster which are distributed along the cluster length. The oligonucleotide primers and the expected length of amplified fragment and the target gene are given in Table 8. All the tested exoconjugants (5 for S. lividans TK24 pUJS+Clavulanic cluster (BamHI) and 5 for S. lividans TK24 pUJS+Clavulanic cluster (Saul) showed positive PCR with fragments of expected length, whereas in the negative control S. lividans TK24 no such fragment was observed.

Table 8: Oligonucleotide primers used for confirmation of transfer of clavulanic acid cluster to S. lividans. Primes were used to confirm complete integration of pUJS+Clavulanic cluster (Saul).

<table>
<thead>
<tr>
<th>Oligonucleotide primer pair</th>
<th>product length</th>
<th>gene amplified</th>
</tr>
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<tbody>
<tr>
<td>AATGATTAAGAGGTAGTTACGAAATGAAACTCATG (SEQ ID NO: 19)</td>
<td>958 bp</td>
<td>sopA: vector backbone</td>
</tr>
<tr>
<td>GGGATACGAGGAGCATTACACATCTGATT (SEQ ID NO: 20)</td>
<td>1261 bp</td>
<td>cas2</td>
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<td>AGTGGTAAGAGGACATCGTGCTAGG (SEQ ID NO: 21)</td>
<td>953 bp</td>
<td>pah2</td>
</tr>
<tr>
<td>TGCACCTCTGCATTATCCCATAG AG (SEQ ID NO: 22)</td>
<td>1849 bp</td>
<td>claR</td>
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<td>GGGCATGTGATGAGGGCATCGAATCGGA (SEQ ID NO: 23)</td>
<td>1439 bp</td>
<td>cypA,ld</td>
</tr>
<tr>
<td>CGCTTCAAGATGACCGTACACCTCCCAAG (SEQ ID NO: 25)</td>
<td>617 bp</td>
<td>ORF21</td>
</tr>
<tr>
<td>CGCCGATATGTCGTTGACCCCTCGGAC (SEQ ID NO: 26)</td>
<td>567 bp</td>
<td>ORF24</td>
</tr>
</tbody>
</table>

Duplication of the cloned clusters in S. clavuligerus ATCC 27064 was confirmed by qPCR copy number determination. Gene copy numbers of pah2, claR and ORF13 genes were determined and the copy number of ceaS1 gene (located elsewhere in the genome and present in one copy in both cases) was used for normalization. Primers and TaqMan MGB probes were designed by Applied Biosystems (Assay By Design service).

Primers and probes sequences were:

SEQ ID NO: 37; Scla claR_F ACGCGGCTCCACATCAC
SEQ ID NO: 38; Scla claR_R GAGCGGCGCAACAG
SEQ ID NO: 39; Scla claR_S CTGCGCCGCCACCC
SEQ ID NO: 40; Scla pah2_F GGGCGATGCGGCAGTAC
SEQ ID NO: 41; Scla pah2_R TGCGCTCGCCTGCC
SEQ ID NO: 42; Scla pah2_S CTGCGCTCGCCTGCC
SEQ ID NO: 43; Scla orf13_F GCGCGCGGCGAGTAC
SEQ ID NO: 44; Scla orf13_R GCGCGCGGCGAGTAC
SEQ ID NO: 45; Scla orf13_S GCGCGCGGCGAGTAC
SEQ ID NO: 46; Scla ceaS1_F CGGCTACATCGAGGCGATT
SEQ ID NO: 47; Scla ceaS1_R GATGCGCAGGAGGAGTGC
SEQ ID NO: 48; Scla ceaS1_S CACCGGCCCTCGAC
Separate 5 µl qPCR reactions for all tested genes were performed for each sample. The reaction mix contained 2.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.25 µl of Gene Expression Assay Mix (Applied Biosystems) and 2 µl of diluted sample DNA. Each sample was analyzed in duplicate of two serial ten-fold dilutions and template-free controls (NTC) were set. The reactions were performed in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using a 384-well thermo block and standard cycling settings. The raw data were normalized to a passive reference dye by the SDS 2.4 (Applied Biosystems) software. Baseline was set automatically and threshold was set manually and Ct values were calculated by the software. Relative quantification was performed using comparative Ct method (ΔΔCt method). Data showed clearly that the genes pah2, claR and ORF13 were present in two copies in the S. clavuligerus ATCC 27064 pUJS+Clavulanic cluster (BamHI) and S. clavuligerus ATCC 27064 pUJS+Clavulanic cluster (Saul) compared to S. clavuligerus ATCC 27064 where one copy of the said genes was measured when data were normalized to ceaS1 gene Ct values, confirming the integration of the BAC vector with clavulanic acid gene cluster to S. clavuligerus.

Example 7: Transfer of cloned clusters to S. clavuligerus and S. lividans with provision of OC31 integrase function in trans

First, a helper vector based on the heat sensitive replicative vector pKC1139 (Kieser et al., 2000, Practical Streptomyces genetics, A laboratory Manual. ISBN-7084-0623-8) was prepared. pKC1139 was cleaved with ScaI and Purified. The Ampicillin, Thioestreptone resistance cassette was amplified from pSET1 5l (Kieser et al., 2000, Practical Streptomyces genetics, A laboratory Manual. ISBN-7084-0623-8) in a PCR reaction using oligonucleotide primers

\[ \text{GCGCGAGCTCTCTGACAGTTACCAATGCTTAATCAGTGAGG (SEQ ID NO: 49) and CCGGGAGCTCTCACTGAGCGAATCGAGGTCAAGGA (SEQ ID NO: 50).} \]

After cloning into a pGEM-T easy PCR cloning vector (Promega) and cleavage with ScaI restriction endonuclease (Promega), 2134 bp fragment was purified and coupled to the ScaI cleaved pKC1139 using T4 DNA ligase. The resulting vector pKC1 139/Amp_Tsr was then cleaved with XbaI restriction nuclease (Promega). The OC31 integrase gene was then amplified from pSET1 52 in a PCR reaction using oligonucleotide primers

\[ \text{GCGCGATCTCAGTACAGTTACCAATGCTTAATCAGTGAGG (SEQ ID NO: 51) and CCGGGAGCTCTCACTGAGCGAATCGAGGTCAAGGA (SEQ ID NO: 52).} \]

After cloning into a pGEM-T easy PCR cloning vector (Promega) and cleavage with XbaI restriction endonuclease (Promega), 1753 bp fragment was purified and coupled to the XbaI cleaved purified and coupled to the ScaI cleaved pKC1 139 using T4 DNA ligase.

The resulting vector pKC1 139/C31, was introduced by transformation into electrocompetent E. coli strain ET1 2567 containing the conjugation helper plasmid pUZ8002 (Paget et al., 1999 J. Bacteriol. 181: 204-211). Conjugation procedure was done as described by Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN-7084-0623-8) using S. clavuligerus ATCC 27064 and S. lividans TK24 spores. KKA-2L agar plates were used for selection of exoconjugants and Thiostrepton (Sigma) was used for selection at 4 µg/mL. Strains S. clavuligerus ATCC 27064 pKC1 139/C31 and S. lividans TK24 pKC1 139/C31 were thus obtained.

Next, pUJS-C31/BamHI, was introduced by transformation into electrocompetent E. coli strain ET1 2567 containing the conjugation helper plasmid pUZ8002 (Paget et al., 1999 J. Bacteriol. 181: 204-211). Conjugation procedure was done as described by Kieser et al., 2000 (Practical Streptomyces genetics, A laboratory Manual. ISBN-7084-0623-8) using S. clavuligerus ATCC 27064 pKC1 139/C31 and S. lividans TK24 pKC1 139/C31 spores. KKA-2L agar plates
were used for selection of exoconjugants and apramycin (Sigma) was used for selection at 60 µg/ml. The experiments were conducted with utmost care that the conjugation conditions were equal for all tested plasmids.

4 colonies were obtained with S. clavuligerus ATCC 27064 pKC1 139/0C31 and 7 colonies with S. lividans TK24 pKC1 139/0C31. PCR and qPCR analysis (methods as in example 5) confirmed the integration of the cloned gene cluster into the genome of both host strains. Strains were then grown in SNK-t medium supplemented with 60µg/ml of Apramycin (inoculated with 1% v/v of spore suspension) and subcultivated for several cycles at 34°C until growth on 4 µg/ml Thiostrepton supplemented KKA-2L agar plates was no longer observed. The strains were cured of the helper plasmid which was additionally confirmed with colony PCR using primers described above (used for amplification of 0C31 integrase).

Relevant literature:

Challis G.L., Hopwood D.A. 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by Streptomyces species. Proceedings of the National Academy of Sciences of the USA, 100: 2; 14555-14561

genruler-mix (avgust, 2011)


Claims

1. A method for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

   (a) Transferring a Bacterial Artificial Chromosome (BAC)-vector into the donor organism, wherein said vector comprises
      (a1) an origin of replication;
      (a2) an origin of transfer;
      (a3) a site-specific recombination system containing a functional integrase and an attP site;
      (a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
      (a5) a selection marker,
   
   wherein said donor organism does not contain a functional attB site

   (b) Isolating the genomic DNA from the donor organism;
   
   (c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;
   
   (d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);
   
   (e) Optionally, isolating said BAC-vector from the prokaryotic organism of step (d);
   
   (f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome.

2. A method for targeted cloning and/or transferring of DNA fragments from a donor organism genome into a host cell, the method comprising the following steps:

   (a) Transferring a BAC-vector into the donor organism, wherein said vector comprises
      (a1) an origin of replication;
      (a2) an origin of transfer;
      (a3) a site-specific recombination system containing an attP site however no functional integrase;
      (a4) a DNA sequence that is substantially homologous to a desired target integration site in the donor organism and that allows for homologous recombination with a corresponding homologous DNA sequence being present in the genomic DNA of the donor organism; and
      (a5) a selection marker,
   
   (b) Isolating the genomic DNA from the donor organism;
   
   (c) Transferring the genomic DNA from step (b), which is present in a BAC-vector derived from the BAC-vector of step (a), into a prokaryotic organism;
   
   (d) Selecting a prokaryotic organism that contains the genomic DNA which is present in the BAC-vector of step (c);
   
   (e) Optionally, isolating said BAC-vector from the prokaryotic organism of step (d);
   
   (f) Introducing the BAC-vector from step (d), or, optionally, the isolated BAC-vector from step (e), via conjugation into the host cell, whereupon said BAC-vector integrates into the host cell genome by action of an integrase the host cell is provided with.
3. The method according to any of the previous claims, wherein the BAC-vector comprises one origin of replication.

4. The method according to any of the previous claims, wherein between any of steps (c) to (f), preferably between steps (c) and (f) the BAC-vector from step (c) is not modified, preferably no attP-site, attP-site integrase cassette, and/or integrase cassette is introduced into said BAC-vector.

5. The method according to any of the previous claims, wherein the genomic DNA that is present in the BAC-vector from steps (c) to (f) comprises a DNA fragment from the donor organism.

6. The method according to claim 5, wherein the DNA fragment has a size of from about 5 kb to about 500 kb.

7. A vector comprising
   (1) an origin of replication;
   (2) an origin of transfer;
   (3) a site-specific recombination system containing a functional integrase and an attP site;
   (4) a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism; and
   (5) a selection marker.

8. A vector comprising
   (1) an origin of replication;
   (2) an origin of transfer;
   (3) a site-specific recombination system containing an attP site however no functional integrase; and
   (4) a selection marker.

9. The vector according to claim 7 or 8, wherein said vector comprises one origin of replication.

10. The vector according to claim 8 or 9, wherein said vector further comprises a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism.

11. A donor organism, comprising a BAC-vector as defined in claims 1 (a1) - (a5), or as defined in any one of claims 7, 9 or 10.

12. The donor organism according to claim 11, wherein said donor organism does not contain a functional attB site.

13. A donor organism, comprising a BAC-vector as defined in claim 2 (a1) to (a5), or as defined in any one of claims 8 to 10.

14. An expression system, comprising
   (1) a host cell, and
   (2) a BAC-vector, wherein said BAC-vector comprises
      i) an origin of replication;
i) an origin of transfer;

ii) a site-specific recombination system containing an attP site and a functional integrase;

iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and

v) a selection marker.

15. An expression system, comprising

(1) a host cell, wherein said host cell is provided with a functional integrase, and

(2) a BAC-vector, wherein said BAC-vector comprises

i) an origin of replication;

ii) an origin of transfer;

iii) a site-specific recombination system containing an attP site however no functional integrase;

iv) a DNA fragment comprising a whole gene cluster or a part of a gene cluster encoding one or more gene product(s) that is (are) part of a biosynthetic pathway for secondary metabolites; and

v) a selection marker.

16. The expression system according to claim 14 or 15, wherein the BAC-vector comprises one origin of replication.

17. A vector comprising the sequence according to SEQ ID NO: 3.

18. A vector comprising the sequence according to SEQ ID NO: 53.

19. The vector according to claim 17 or 18, additionally comprising a DNA sequence that allows for homologous recombination with a corresponding substantially homologous DNA sequence being present in the genomic DNA of a donor organism.

20. Use of a vector according to any of claims 17 to 19 for cloning and/or transfer of a DNA fragment, and/or for expression of said DNA fragment.
Restriction of genomic DNA, self-ligation, transformation of E. coli and selection of ApI^R clones.
LOCUS pBeloBAC11+pSET152 10789 bp DNA circular 21-MAY-2012
DEFINITION Cloning vector pBeloBAC11+pSET152, complete sequence.
SOURCE Cloning vector pBeloBAC11.
ORGANISM Cloning vector pBeloBAC11
other sequences; artificial sequences; vectors.

FEATURES Location/Qualifiers
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  /vntlifique=60"
CDS complement(4525..5184)
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  /vntlifique=33"
  /label=Rep_Origin_1
  /note="ori2 (oriS)"
gene 6524..7279
  /gene="repE"
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1201 cgccgagctat ctggccgcccc ccgatagct tcagaatgcgc cccgatgcgg cc
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1741 ctggctgcttgtc ggctgctttg ccggcctttg cttaacattgct
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DEFINITION Cloning vector pBeloBAC11+pSET152-C31, complete sequence.

SOURCE Cloning vector pBeloBAC11.

ORGANISM Cloning vector pBeloBAC11
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DEFINITION Cloning vector pBAC_Lek, complete sequence.

SOURCE Cloning vector pBeloBAC11.

ORGANISM Cloning vector pBeloBAC11
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INV. C12N15/70
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, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
* "A" document defining the general state of the art which is not considered to be of particular relevance
* "E" earlier application or patent but published on or after the international filing date
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* "O" document referring to an oral disclosure, use, exhibition or other means
* "P" document published prior to the international filing date but later than the priority date claimed
* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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* "Z" document member of the same patent family

Date of the actual completion of the international search: 4 October 2013
Date of mailing of the international search report: 17/10/2013

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

Young, Craig

Authorized officer: 

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
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