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(54) Title: MODIFIED BACTERIOPHAGE

(57) Abstract: A modified bacteriophage capable of infecting a plurality of different target bacteria, which bacteriophage includes a toxin gene encoding a toxin protein which is toxic to the target bacteria; wherein the bacteriophage is lytic; and wherein the bacteriophage expresses host range determinant proteins which have a plurality of bacterial host specificities.

MODIFIED BACTERIOPHAGE

The present invention relates to a modified bacteriophage, uses thereof, and compositions containing the modified bacteriophage.

Background to the Invention

The World Health Organisation's 2014 report on global surveillance of antimicrobial resistance reveals that antibiotic resistance is a global problem that is jeopardising the ability to treat common infections in the community and hospitals. Without urgent action, the world is heading towards a post-antibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill (WHO, 2014). Antibiotic resistance complicates patients' recovery from even minor operations and is increasingly causing treatment failures. In fact, there are now strains of some genera of bacteria circulating globally which are resistant to all available antibiotics. Such strains commonly fall within the scope of the so-called ESKAPE pathogens – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and Enterobacter species (Boucher *et al.*, 2009). The term ESKAPE pathogens was coined by Boucher *et al.*, to emphasize that these bacteria currently cause a majority of hospital infections in the US and Europe and can effectively "escape" the majority, if not all, available antibiotics with panantibiotic-resistant infections now occurring. The death rate for patients with serious infections caused by common bacteria treated in hospitals is approximately twice that of patients with infections caused by the same non-resistant bacteria, e.g. people with methicillin-resistant *Staphylococcus aureus* (MRSA) infections are estimated to be 64% more likely to die than people with a non-resistant form of the infection (WHO, 2014). Of the Gram positive bacteria, methicillin resistant *S. aureus* continues to be a major cause of morbidity and mortality in hospitals in the US and Europe. However, in more recent years, several highly resistant Gram negative pathogens, including *Acinetobacter* species, multidrug resistant (MDR) *P. aeruginosa*, and carbapenem-resistant *Klebsiella* species and *Escherichia coli*, have emerged as major pathogens causing serious, and sometimes untreatable, infections. Advances in medicine mean that increasingly complex procedures take place: and these advances are leading to a growing number of elderly patients and patients undergoing surgery, transplantation, and chemotherapy all of which will produce an even greater number of

immunocompromised individuals at risk of these infections (Walker *et al.*, 2009). This phenomenon has led to a greater dependence on, and requirement for, effective antibiotics.

P. aeruginosa is one bacterium which is frequently multi-drug resistant (MDR) having intrinsic resistance due to low permeability of its outer membrane limiting drugs getting into the cell, and a multitude of efflux pumps to expel any drugs that successfully manage to enter the cell. *P. aeruginosa* is also acquiring additional resistance mechanisms, including resistance to the "antibiotics of last resort" for Gram negatives, the carbapenems. *P. aeruginosa* causes approximately 10% of all hospital acquired infections and is the second leading cause of hospital-acquired pneumonia, which accounts for 50 % of all hospital-acquired infection prescribing. *P. aeruginosa* infections in hospitals commonly require intravenous (IV) treatment with current standard of care for *P. aeruginosa* infections dictating that patients are treated with at least two antibiotics. Unfortunately, resistance frequently develops in patients during therapy. With so few new classes of antibiotic developed and approved for market within the last 30-40 years, there is a critical need for novel, safe and effective antibacterial agents.

As an alternative to conventional antibiotics, one family of proteins which demonstrate broad spectrum antibacterial activity inside bacteria comprises the α/β-type small acid-soluble spore proteins (known henceforth as SASP). Inside bacteria, SASP bind to the bacterial DNA: visualisation of this process, using cryoelectron microscopy, has shown that SspC, the most studied SASP, coats the DNA and forms protruding domains and modifies the DNA structure (Francesconi *et al.*, 1988; Frenkiel-Krispin *et al.*, 2004) from B-like (pitch 3.4 nm) towards A-like (3.18 nm; A-like DNA has a pitch of 2.8 nm). The protruding SspC motifs interact with adjacent DNA-SspC filaments packing the filaments into a tight assembly of nucleo-protein helices. In 2008, Lee *et al.* reported the crystal structure at 2.1 Å resolution of an α/β-type SASP bound to a 10-bp DNA duplex. In the complex, the α/β-type SASP adopt a helix-turn-helix motif, interact with DNA through minor groove contacts, bind to approximately 6 bp of DNA as a dimer and the DNA is in an A-B type conformation. In this way DNA replication is halted and, where bound, SASP prevent DNA transcription. SASP bind to DNA in a non-sequence specific manner (Nicholson *et al.*, 1990) so that mutations in the bacterial DNA do not affect the binding of SASP. Sequences of α/ß-type SASP may be found in appendix 1 of WO02/40678, including SASP-C from *Bacillus megaterium* which is the preferred α/ß-type SASP.

WO02/40678 describes the use as an antimicrobial agent of bacteriophage modified to incorporate a SASP gene. In order to provide effective production of the modified bacteriophage in a bacterial host, WO02/40678 aims to avoid factors which may disrupt proliferation of the production host, such as expression of the SASP gene during production. To this end, the SASP gene was put under the control of an inducible promoter. In a preferred arrangement, at least one of the gene encoding products involved in the lytic process was inactivated.

WO2009019293 describes that effective production of bacteriophage may be achieved where the bacteriophage has been modified to delete a lysis gene and to carry a gene encoding a SASP under the control of a promoter which is controlled independently of the bacteriophage, and which is constitutive with no exogenous or *in trans* regulation necessary or provided. Such a modified bacteriophage is maintained as a prophage in a manufacturing host strain, and may be amplified by suitable induction methods to synthesise new phage within the manufacturing host strain. In this arrangement, the manufacturing host strain must be lysed by the addition of exogenous substances, e.g. lytic enzymes or chemicals, in order to release viable phage. An example of a suitable promoter is the *fbaA* promoter from *S. aureus* which is used to drive expression of the SASP-C gene from *Bacillus megaterium* and which, when present in multiple copies, for example following infection of target cells, drives toxic levels of SASP expression.

Bacteriophage vectors modified to contain a SASP gene have generally been named SASPject vectors. Once the SASP gene has been delivered to a target bacterium, SASP is produced inside those bacteria where it binds to bacterial DNA and changes the conformation of the DNA from B-like towards A-like. Production of sufficient SASP inside target bacterial cells causes a drop in viability of affected cells.

Bacteriophage have been used as medicines for the treatment of bacterial infections since the 1920s or 30s. Generally, bacteriophage are specific to their bacterial host. Some bacteriophage are temperate and others non-temperate. Temperate phage are able to infect the host cell and integrate into the host cell genome becoming a prophage which is generally harmless to the host cell in this state. Non-temperate or "lytic" phage are only able to replicate in a lytic lifestyle by making new bacteriophage progeny and ending in lysis of the host cell and release of mature phage particles. For useful medicines, the challenge is to provide bacteriophage

compositions which can be used to treat infection from a variety of different bacteria in an effective way. It is commonly thought that this is achieved using the most potent bacteriophage compositions available: those with a broadened host range, possibly as a mixture or "cocktail" of bacteriophage (Carlton, 1999; Kutateladze and Adamia, 2010). Cocktails of wild type phage have been used to ensure sufficient spectrum of activity against clinical strains of bacteria (Burrowes and Harper, 2012). Such cocktails can consist of up to 20 different and unrelated phage (Abedon 2008). As an alternative to the cocktail approach, *E. coli* bacteriophage K1-5 has been isolated. This is a naturally-occurring obligately lytic phage which carries more than one host range determinant (HRD) allowing it to infect and replicate on both K1 and K5 strains of *E. coli* (Scholl *et al*, 2001). These phage are considered to be extra potent.

There remains a need to provide improved bacteriophage for use in treating bacterial infections in medicine as well as inhibiting or preventing bacterial cell growth in medical and non-medical situations.

Summary of the Invention

In a first aspect, the present invention provides a modified bacteriophage capable of infecting a plurality of different target bacteria, which bacteriophage includes a toxin gene encoding a toxin protein which is toxic to the target bacteria; wherein the bacteriophage is lytic; and wherein the bacteriophage expresses host range determinant (HRD) proteins which have a plurality of bacterial host specificities. The bacterial host specificity of the HRD is advantageously within the same bacterial species.

It has surprisingly been found that a modified bacteriophage may be produced which is capable of infecting a variety of different target bacteria, and which is effective for use in medicine when engineered to carry a gene for a toxic protein. The modified bacteriophage is lethal to bacterial cells, and, despite being lytic, and thus requiring completion of the phage lifecycle to be released from cells, can be manufactured in a host cell through several rounds of infection and replication.

In one aspect, the HRD proteins which have a plurality of bacterial host specificities are hybrid HRD proteins each comprising an amino acid sequence from a plurality of different bacteriophages. Because the modified bacteriophage expresses a hybrid HRD protein this

confers an enhanced host range on the phage. Bacteriophages according to the invention may be produced by genetic engineering, for example by selecting HRD from closely related phage. Having created such an extra-potent lytic phage, it can then be engineered to create a vector incorporating the toxin gene, which vector is capable of being manufactured in a host cell and effective as an antibacterial agent *in vivo*.

In another aspect, the HRD proteins expressed by the bacteriophage comprise a plurality of different HRDs, wherein each HRD has a different bacterial host specificity. In this aspect the HRDs can be homologous, heterologous, (hybrid) or a mixture of homologous and hybrid HRDs. The plurality of different HRDs confers upon the bacteriophage an enhanced host range. Such bacteriophage may be produced by genetic engineering, for example by selecting HRDs from phage which infect the same bacterial species. Having created such an extra-potent lytic phage, it can then be engineered to create a vector incorporating the toxin gene, which vector is capable of being manufactured in a host cell an effective as an antibacterial agent *in vivo*.

In a preferred arrangement, the toxin gene comprises an α/β small acid-soluble spore protein (SASP) gene encoding a SASP. In one aspect, the term 'SASP' as used in the specification refers to a protein with α/β-type SASP activity, that is, the ability to bind to DNA and modify its structure from its B-like form towards A-like form, and not only covers the proteins listed in appendix 1 of WO02/40678, but also any homologues thereof, as well as any other protein also having α/β-type SASP activity. In an alternative aspect, the term 'SASP' as used in the specification refers to any protein listed in appendix 1 of WO02/40678, or any homologue having at least 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 98% or 99% sequence identity with any one of the proteins. listed in appendix 1 of WO02/40678. In another alternative aspect, the term 'SASP' as used in the specification refers to any protein listed in appendix 1 of WO02/40678.

The SASP gene may be chosen from any one of the genes encoding the SASP disclosed in Appendix 1 of WO02/40678. In a preferred arrangement the SASP is SASP-C. The SASP-C may be from *Bacillus megaterium*.

It is preferred that the SASP gene is under the control of a constitutive promoter which is

advantageously sufficiently strong to drive production of toxic levels of SASP when the modified bacteria phage is present in multiple copies in the target bacterium. Useful constitutive promoters include *pdhA* for pyruvate dehydrogenase E1 component alpha sub units, *rpsB* for the 30S ribosomal protein S2, *pgi* for glucose-6-phosphate isomerase and the fructose bisphosphate aldolase gene promoter *fda*. Preferred regulated promoters, active during infection, are *lasB* for elastase. These promoters are typically from *P. aeruginosa*. Promoters having a sequence showing at least 90% sequence identity to these promoter sequences may also be used.

The present invention is generally applicable to bacteriophage infecting a variety of different target bacteria. In one arrangement at least one of the target bacteria is *Pseudomonas*. Advantageously, the plurality of different target of bacteria is a plurality of different *Pseudomonas* bacteria. An important target is *Pseudomonas aeruginosa*.

It was previously considered that an obligate lytic phage would be unsuitable for use as a SASPject vector, since a requirement of a SASPject vector is that it is specifically not lytic for optimal therapeutic use. A non-lytic vector allows an increased time window for SASP expression, thereby increasing the efficacy of the treatment. In addition, prevention of rapid lysis upon treatment *in vivo*, was considered advantageous because it would limit the potential release of antibiotic resistance genes and toxic cell wall components which can lead to a dangerous inflammatory response.

The approach described in the present invention is advantageous as compared to the phage cocktail approach described previously. Mixtures of modified bacteriophage, such as SASPject vectors, are identical in structure and genome sequence, other than carrying a different HRD or hybrid HRD. One advantage is that control of the manufacturing process for the mix of SASPjects will be straightforward, which is an important aspect of a pharmaceutical preparation: the process will be materially the same for phage modified to carry a heterologous HRD as they share identical or near-identical biophysical properties. Another advantage is that the *in vivo* characteristics of the SASPject vectors are likely to be similar, e.g. pharmacokinetics/pharmacodynamics, as each vector is structurally the same or similar.

In one aspect of the present invention it has been found that phage can be created which are extra-potent obligately-lytic bacteriophage carrying HRDs which have a plurality of bacterial

host specificities. Surprisingly, such phage can be used to make enhanced SASPject vectors which retain a lytic phenotype, and remain effective *in vivo*, and such phage can be manufactured to an adequate titre in a bacterial host. WO02/40678 and WO2009019293 have taught the creation of SASPject vectors from bacteriophage which have been modified to remove one or more lysis genes, and which reside as prophage in a host cell. Such SASPject vectors are manufactured by prophage induction and the cells are lysed by the exogenous addition of lysis agents, e.g. cell wall degrading enzymes or chemicals such as chloroform. As such, the cells do not need to remain viable until the end of the phage lifecycle, as the final lysis step is not reliant upon the synthesis and accumulation in the host cell of the modified phage's own lysis proteins. Given the toxic nature of the SASP protein, and the extra-potent nature of the modified phage described in the present invention, it was not anticipated that such phage would be suitable for modification to create SASPjects which could be manufactured via an obligate lytic lifecycle, requiring the phage to complete its lytic lifecycle in order to create viable SASPject vectors. However, it has been found that such SASPject vectors are capable of replication in host cells to an adequate titre. This allows the manufacture of SASPject vectors in a quantity suitable for effective *in vivo* use. Advantageously, SASPject vectors based upon such modified obligate lytic phage are capable of replication at the site of infection. This means that the dose required for a lytic SASPject can be lower than that required for a non-lytic SASPject when used *in vivo*. Lytic SASPject vectors according to the invention may be considered more potent than their non-lytic counterparts.

Phage suitable for such modification may be isolated by screening for phage capable of infecting a chosen bacterial species. For instance, phage may be isolated which infect *Pseudomonas aeruginosa*, by screening environmental sources for phage which are able to form plaques on representative *P. aeruginosa* strains (Gill and Hyman, 2010). Isolated phage may have their whole genomes sequenced and annotated. HRDs may be tail fibre proteins, which are commonly found to be proteins responsible for the initial recognition/binding to the host bacterium, for instance in phage T4, T5 and T7 (Rakhuba *et al.*, 2010). Alternatively other HRD may be baseplate proteins. Phage genomes may be searched for potential HRD sequences by assessing the homology of all proteins in the phage genome to known sequences, using BLAST searches.

According to the present invention it is preferred that each HRD has a broad host range. This may be defined as the ability to infect >50% of a diverse collection or clinical isolates, totalling

at least 35, preferably at least 40, more preferably at least 44, and most preferably >50 in number. Such isolates should be from a range of geographical locations, including Europe, the Americas, and Asia, should carry a diverse range of antibiotic resistance phenotypes, including multi-drug resistant (MDR) strains, and should be from a diverse range of infection sites, such as strains cultured from blood, lung and skin infections. Such isolates can be obtained from public strain collections such as the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC). Generally, each tail fibre protein comprises a C-terminal receptor binding region for binding to the target bacteria and an N-terminal region linking the C-terminal receptor binding region to the body of the bacteriophage. Each of the C-terminal and N-terminal regions may be from different bacteriophage. In one arrangement, the N-terminal region comprises amino acids 1 to 628 of the tail fibre protein and the C-terminal region comprises the amino acids 629 to 964 of the tail fibre protein.

The C-terminal region may have no more than 96% amino acid sequence identity with the C-terminal region of bacteriophage Phi33 and may be from any one of the bacteriophage Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH-4, PTP47, C36, PTP92 and PTP93. Lower amino acid sequence identities in the C-terminal region are preferred. Advantageously the sequence identity is less than 90%, more advantageously less than 80%, preferably less than 70%, more preferably less than 60%, still more preferably less than 50%, particularly preferably less than 40%, more particularly preferably less than 30%. The N-terminal region may have at least 90% and advantageously at least 95% amino acid sequence identity with the N-terminal region of bacteriophage Phi33 and may be from any one of bacteriophage Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH-4, PTP47, C36, PTP92 and PTP93. The N-terminal region and the C-terminal region may be from the same bacteriophage to provide a homologous tail fibre protein. Alternatively, the N-terminal region and the C-terminal region may be from different bacteriophage tail fibre proteins to provide a heterologous tail fibre protein. In one arrangement where the phage tail fibre protein is homologous, each tail fibre protein is from a bacteriophage selected from Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH-4, PTP47, C36, PTP92 and PTP93.

It is advantageous to identify phage tail fibre proteins which share sequence identity of greater than 90% in the N-terminal region. For example several phage – Phi33, PTP47, PTP92 and C36 – with a broad host range for *P. aeruginosa* strains (all of these phage infect >60%, when analysed against 260 strains), have been isolated/identified and their genomes sequenced.

Analysis of the genome sequences of Phi33, PTP47, PTP92 and C36 reveals that they contain genes encoding putative tail fibre proteins with a high level of sequence identity in the N-terminal region (>95% amino acid sequence identity), following a 2 sequence BLAST alignment, compared to the Phi33 tail fibre amino acids 1-628 (amino acid identity in parentheses): C36 (96%), PTP47 (98%), PTP92 (97%). BLAST searches have shown that these 4 phages are related to 10 other deposited phage genome sequences which, together, form the family of PB1-like phage: PB1, SPM1, F8, LBL3, KPP12, LMA2, SN, JG024, NH-4, 14-1 (Ceyssens *et al.*, 2009). The homology of these putative tail fibre proteins was assessed. Following a 2 sequence BLAST alignment, compared to the Phi33 tail fibre protein (amino acid identity in parentheses): LBL3 (96%), SPM-1 (95%), F8 (95%), PB1 (95%), KPP12 (94%), LMA2 (94%), SN (87%), 14-1 (86%), JG024 (83%), NH-4 (83%), C36 (96%), PTP47 (86%), PTP92 (83%). An alignment of all 14 of the aforementioned phage tail fibre proteins is shown in Figure 13.

Analysis of the annotated tail fibre protein sequences from these 14 phages reveals that the N-terminal region of the proteins - equivalent to Phi33 tail fibre amino acids 1-628 – show an even higher level of sequence identity at the amino acid level than the sequence identity of these proteins over their entire length, in the range of 96-100% for all 14 proteins. Following a 2 sequence BLAST alignment, compared to the N-terminal amino acids 1-628 of the Phi33 tail fibre protein (amino acid identity in parentheses): LBL3 (96%), SPM-1 (96%), F8 (96%), PB1 (96%), KPP12 (98%), LMA2 (99%), SN (99%), 14-1 (97%), JG024 (97%), NH-4 (97%), PTP47 (98%), C36 (96%), PTP92 (97%). However, the C-terminal region of the protein – equivalent to Phi33 tail fibre amino acids 629-964 – is not as conserved as the N-terminal region in some of the proteins, the range of sequence identity being typically 57-96%. Following a 2 sequence BLAST alignment, compared to the C-terminal 629-964 amino acids of the Phi33 tail fibre protein (amino acid identity in parentheses): LBL3 (94%), SPM-1 (93%), F8 (93%), PB1 (94%), KPP12 (87%), LMA2 (85%), SN (65%), 14-1 (65%), JG024 (57%), NH-4 (57%), PTP47 (64%), C36 (96%), PTP92 (57%). Analysis of phage tail fibres from other, well characterised, phage has shown that they possess an N-terminal tail base plate binding region and a C-terminal receptor binding region (Veesler and Cambillau, 2011). In experimental analysis of their bacterial strain host range, using plaque assay or growth inhibition tests, the phage Phi33, PTP47, PTP92 and C36 have overlapping but non-identical host range (Table 1). Taken together with the established evidence for the role of the C-terminal region of phage tail fibres being involved in bacterial host receptor binding, and the

sequence variation in the C-terminal region of these 4 phage, and their similar but non-identical host range, it is postulated that the C-terminal variation is associated with host range in the phage assessed.

It is further provided, according to this invention, that the genes for a homologous tail fibre protein can be taken from one phage and added to another, replacing the resident tail fibre, based upon their high level of sequence identity in the N-terminal region. The N-terminal region is thought to be involved in the binding of the tail fibre to the phage tail (Veesler and Cambillau, 2011), allowing the formation of viable phage with the host range associated with donor phage's tail fibre. Hybrid tail fibre genes may be made, carrying the conserved N-terminal tail attachment region of the tail fibre from a recipient phage, together with the variable C-terminal receptor-binding region from a homologous donor phage tail fibre protein, using tail fibres genes such as those described herein. Such tail fibre hybrid genes could be used to replace the tail fibres of the phage. This provides an N-terminal region of the hybrid tail fibre (from the recipient phage) and allows the formation of viable phage with the host range associated with donor phage's tail fibre C-terminal receptor-binding region. Transplantation of engineered tail fibre hybrid genes into a recipient phage has been demonstrated in the present invention. Using standard molecular genetic techniques, Phi33 has been modified to carry heterologous tail fibre hybrids from the following phage: PTP92, PTP47, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, NH-4. All modified phage have been shown to be viable and able to plaque on *P. aeruginosa*. (The nomenclature of tail fibre hybrids is as follows: As an example, a hybrid gene such that the N-terminal tail attachment region of Phi33 is hybridised with the C-terminal receptor binding region of PTP47 is Phi33(N)PTP47(C).)

In one such modified phage, Phi33 was engineered such that its tail fibre gene carries the C-terminal receptor binding region of PTP92, creating PTP93 (Phi33(N)PTP92(C)). This was assessed in more detail, by testing the host range against 35 diverse *P. aeruginosa* clinical isolates. Comparing host range of the progenitor phage (Phi33), the tail fibre donor (PTP92) and the hybrid phage (PTP93), the host range of the PTP93 hybrid phage is equivalent to that of the tail fibre donor phage (PTP92) rather than Phi33, but it was surprisingly found that in some instances PTP93 possesses the host range of Phi33 on strains that PTP92 cannot infect, thus PTP93 inherits the host range of both phages (Table 2). PTP93 possesses a broader host range (92%) than either Phi33 (74%) or PTP92 (66%) (Table 2). PTP93 is an example of an obligately lytic bacteriophage which can be considered as "extra-potent" as it possesses a

characteristic above and beyond those exhibited in their unmodified state. Such extra potent phage are suitable for further modification to make SASPject vectors.

Phi33, through assessment of sequence homology by one skilled in the art, can be placed in the group of PB1-like phage (Ceyssens *et al.*, 2009), and may be considered a broad host range phage by the definition given earlier. Such a phage could be isolated by the methods described earlier. Phi33 is suitable for genetic modification to introduce a gene for SASP under the control of a promoter. Such a modified phage would be suitable for use in treating infections caused by susceptible *P. aeruginosa* bacteria, either on its own or in combination with other modified bacteriophage. In one arrangement, the invention may not extend to an isolated bacteriophage PTPX31 or PTPX32.

A preferred approach according to the present invention is to use one or more obligately lytic phage, each engineered to carry a SASP gene expressed from a constitutive promoter, each phage being genetically identical other than their tail fibre or tail fibre hybrid gene. Preferred obligately lytic phage for modification and for provision of tail fibre genes to create tail fibre genes or tail fibre hybrid genes are phages carrying tail fibre genes which encode predicted proteins that possess ≥90% amino acid sequence identity in their N-terminal regions compared to N-terminal regions of the tail fibre of other isolated or identified phage.

Another preferred approach according to the present invention is to use one or more obligately lytic phage engineered to express 2 or more HRDs (extra potent obligately lytic phage), each engineered to carry a SASP gene expressed from a constitutive promoter, each phage being genetically identical other than carrying different tail fibre genes, or tail fibre hybrid genes, Preferred obligately lytic phages for modification and for provision of tail fibre genes to create phages carrying multiple tail fibre genes or tail fibre hybrid genes are phages carrying tail fibre genes which encode predicted proteins that possess ≥90% amino acid sequence identity in their N-terminal regions compared to N-terminal regions of the tail fibre of other isolated or identified phage.

Preferred obligate lytic phage meeting these criteria are Phi33, PTP92, PTP47, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, NH-4, PTP93, JG024, PTP47 and C36. Such phage can be identified by a simple PCR assay, by subjecting plaques of isolated phage to PCR with primers specific to highly conserved regions in the N-terminal region of the tail genes. In such

a way, suitable phage can be identified without whole genome sequencing. Phage PB1 can be obtained from a public strain collection. Phages need not be isolated or provided in order to generate tail fibre sequences as such sequences may be identified in DNA sequence databases, or other sources of DNA sequences, which may provide the information necessary in order to synthesise and clone, by standard methods, such sequences, or to create hybrid tail fibre sequences.

Particularly preferred phage for modification are PTP93, Phi33, PTP92, PTP47 and C36. Particularly preferred extra-potent obligate lytic phage are: Phi33, modified to carry the tail fibre hybrid Phi33(N)PTP92(C) in place of the resident tail fibre; Phi33 modified to carry the tail fibre hybrid Phi33(N)PTP47(C) in place of the resident tail fibre. In one aspect of the present invention, preferred extra-potent non-lytic SASPject derivatives of Phi33 include: Phi33, modified to carry the tail fibre hybrid Phi33(N)PTP92(C) in place of the resident tail fibre and carrying SASP-C from *Bacillus megaterium*, codon-optimised for expression in *P. aeruginosa*, under the control of the *P. aeruginosa* fructose bisphosphate aldolase (*fda*) gene promoter; Phi33 modified to carry the tail fibre hybrid Phi33(N)PTP47(C) in place of the resident tail fibre and carrying SASP-C from *Bacillus megaterium*, codon-optimised for expression in *P. aeruginosa*, under the control of the *P. aeruginosa* fructose bisphosphate aldolase (*fda*) gene; Phi33 modified to carry SASP-C from *Bacillus megaterium*, codon-optimised for expression in *P. aeruginosa*, under the control of the *P. aeruginosa* fructose bisphosphate aldolase (*fda*) gene promoter. In a particularly preferred aspect the present invention provides a mixture of SASPject comprising or consisting of the 3 aforementioned SASPjects formulated together.

A mixture of three modified bacteriophage, designated PT3.9, was constructed and its efficacy in killing *P. aeruginosa* tested. The mixture consists of: Phi33 carrying the Phi33(N)PTP92(C) tail fibre, modified to carry the *fda*-SASP-C (*P. aeruginosa* codon optimised sequence) (PTP388); Phi33 carrying the Phi33(N)PTP47(C) tail fibre, modified to carry the *fda*-SASP-C (*P. aeruginosa* codon optimised sequence) (PTP389); Phi33 carrying the Phi33(N)Phi33(C) tail fibre, modified to carry the *fda*-SASP-C (*P. aeruginosa* codon optimised sequence) (PTP387).

The efficacy of PT3.9 was tested in a 6 hour time-kill curve experiment against a multi-drug resistant (MDR) clinical isolate (trachea isolation site, antibiotic resistance to ceftazidime,

piperacillin-tazobactam and imipenem) of *P. aeruginosa*, strain 3503 and reference strain ATCC 27853. Briefly, cultures were set up in Luria Bertani (LB) broth supplemented with 5 mM calcium chloride, 5 mM magnesium sulphate and 0.1% glucose (LC broth), and grown at 37 °C. 5×10^5 colony forming units per millilitre (cfu/ml) of *P. aeruginosa* was incubated with 3×10^9 plaque forming units per ml (pfu/ml) of PT3.9, or extra LC broth as a control (untreated culture). Samples were removed at 0, 1, 2, 4, and 6 hours for serial dilution and plating on LC agar plates and then overnight incubation at 32 °C. For both strains, the viable cell count was reduced from 5×10^5 cfu/ml to below the limit of detection (10^2 cfu/ml) within 1 hour of treatment, and no viable cells were detected after 6 hours (Figure 14). In contrast, the untreated control culture grew to between 5×10^8 and 1×10^9 cfu/ml for both strains. This demonstrates the ability of PT3.9 to kill clinical strains of *P. aeruginosa*.

PTP387, a component SASPject of PT3.9, was tested *in vivo* in comparison to PTP284, a SASPject which is equivalent to PTP387 other than lacking one of the lysis genes (endolysin). An immunocompetent murine model of *P. aeruginosa* bacteraemia was used to assess the potency of the two SASPject vectors. Briefly, animals were infected by intravenous injection with an incoculum of 7.5 $\log_{10}$ cfu of *P. aeruginosa* strain ATCC 27853. At 2 hours post infection, the mice were treated either with PTP284, PTP387, vehicle (buffer), or ceftazidime (50 mg/kg) by intravenous injection. PTP284 and PTP387 were administered at the following doses: 3×10^{10}, 1×10^{10}, 3×10^9, and 1×10^9 pfu. At 22 hours post treatment, mice were euthanised by carbon dioxide asphyxiation, and the livers were removed and weighed. Liver tissue was homogenised in tryptone soya broth (TSB) and the number of viable cells in the liver tissue was enumerated by serial dilution and plating on Cetrimide agar plates. A control (not treated) group of mice was euthanised at 2 hours post infection and the viable cells in the liver tissue enumerated, to assess the viable cell count in the lung tissue at the time of treatment. A dose response was seen for both PTP284 and PTP387, when assessing the geometric mean of the bioburden levels of *P. aeruginosa* in liver tissue (Figure 15). The potency of PTP387 was greater than that of PTP284 when assessing the log reduction in cfu/g liver tissue compared to the level assessed at the time of treatment: 22 hours post treatment, PTP387 caused a 3-log reduction in liver tissue bioburden at a 1×10^9 pfu dose, whilst an equivalent effect of PTP284 was seen at 1×10^{10} pfu. PTP387 caused a greater maximum effect on liver tissue bioburden reduction compared to PTP284: at a dose of 3×10^{10} pfu, PTP387 caused a 5.4-log reduction in liver tissue bioburden at 22 hours post treatment, whilst PTP284 caused a 2.4-log reduction.

This demonstrates the increased potency of the lytic SASPject *in vivo* when compared to equivalent non-lytic SASPject.

The mixture of lytic modified SASPject phage comprising PT3.9 was tested *in vivo* in comparison to the mixture of non-lyitc modified SASPject phage comprising PT3.8. The modified phage in PT3.8 are identical to those in PT3.9 other than lacking the lysis gene (endolysin) present in PT3.9. A neutropenic murine model of *P. aeruginosa* pneumonia was used to assess the potency of the two mixtures of SASPject phage. Briefly, animals were rendered neutropenic by immunosuppression with cyclophosphamide 200mg/kg 4 days before infection and 150mg/kg 1 day before infection by intraperitoneal injection. Animals were anaesthetised and infected intranasally with 4.5×10^3 cfu of *P. aeruginosa* strain ATCC 27853. Groups of 6 mice were given each treatment. PT3.8 and PT3.9 were administered via the trachea 15 minutes post infection in an aerosolised form at the following doses: 1×10^9, 3×10^9, 1×10^{10} and 1.5×10^{10} pfu. Tobramycin (200µg) was administered in an aerosolised form to a control group of mice, and another group was treated with placebo buffer. Animals were euthanised 24 hours post treatment and lung tissue was removed and homogenised in phosphate buffered saline (PBS). The tissue homogenate was serially diluted and plated on agar plates to enumerate the bacteria in the lung tissue. A control (not treated) group of mice was euthanised at 15 minutes post infection and the viable cells in the lung tissue enumerated, to assess the viable cell count in the tissue at the time of treatment. A dose response was seen for both PT3.8 and PT3.9, when assessing the geometric mean of the bioburden levels of *P. aeruginosa* in lung tissue (Figure 16). The potency of PT3.9 was greater than that of PT3.8. When assessing the log reduction in cfu/g lung tissue compared to the level assessed at the time of treatment: 24 hours post treatment, PT3.9 caused a > 2-log reduction in lung tissue bioburden at a 1×10^9 pfu dose, whilst an equivalent effect of PT3.8 was seen at doses between 1×10^{10} and 1.5×10^{10} pfu. Thus PT3.9 was more than 10 times more potent than PT3.8.

The ability of PTP387 to propagate in a host cell and yield bacterial lysates suitable for further purification was assessed in comparison to the unmodified phage (Phi33). Briefly, overnight culture of *P. aeruginosa* strain 1868 were used to inoculate 2 X 1 L bioreactor vessels, each containing 0.5 L of rich broth medium, to an OD600 of 0.05. The cultures were grown to OD600 = 0.3-0.4 and then infected with phage Phi33 or PTP387 to a final concentration of 1×10^7 pfu/ml, and grown further at 37°C. After 5 hours of growth, the cultures were treated

with benzonase and sterile filtered. The concentration of each phage was determined by plaque assay. The lysate titres yielded for PTP387 were comparable to those obtained for Phi33: $2x10^{11}$ pfu/ml (PTP387) and $5x10^{11}$ pfu/ml (Phi33). The titre of PTP387 increased by 4-logs over 5 hours, demonstrating the ability of this phage to complete several cycles of infection, multiplication and lysis, despite the presence of the toxic SASP gene.

Fermentations were performed at 15.5-16L scale for all 3 of the modified phage which comprise PT3.9. Briefly, overnight cultures of *Pseudomonas aeruginosa* were used to inoculate a 16L bioreactor containing 15.5 to 16 L of rich broth medium, to an OD600 of 0.05. Each culture was grown to OD600 = 0.4-0.7 before being infected with either PTP387, PTP388 or PTP389 (SASPject phage which comprise PT3.9) to a final concentration of 1×10^7 pfu/ml. Cultures were grown for another 5 hours before Benonzase treatment. Samples were removed, sterile filtered, and analysed by plaque assay. Two fermentations were performed for each SASPject phage. All 3 of the modified SASPject phage comprising PT3.9 were able to propagate to 5×10^{11} to 1×10^{12} pfu/ml. Thus all 3 of the modified SASPject phage are able to complete several cycles of infection, multiplication and lysis, despite the presence of the toxic SASP gene.

The quantities of PTP387 obtained by such manufacturing processes at 1L scale are suitable for effective *in vivo* use, as demonstrated by the use of such preparations in murine infection models (Figure 15 and 16). The levels of PT387, PTP388 and PTP389 obtained by such manufacturing processes at 15.5-16L scale are suitable for effective *in vivo* use, as demonstrated by the use of such preparations, when combined as a mixture (PT3.9), in murine infection models (Figures 15 and 16).

In another embodiment, an obligately lytic phage may be modified to create a SASPject by inserting a SASP gene under the control of a constitutive promoter, and the tail fibre gene could be deleted altogether. Such phage must be propagated in a strain in which a tail fibre gene or tail fibre hybrid gene is expressed *in trans*. In such an instance, the SASPject progeny from such a strain would carry a single tail fibre, derived from the propagation strain, yet would lack in their genomes any tail fibre or tail fibre hybrid gene(s). Several such propagation strains could be constructed and the same tail fibre deleted SASPject expressed in each. In this way several different SASPject derivatives could be made, each carrying a different tail fibre or tail fibre hybrid protein. These SASPjects could be used to formulate a mixture.

In another embodiment, an obligately-lytic phage engineered to carry a SASP gene expressed from a constitutive promoter may be propagated in a host strain carrying the gene(s) for hybrid tail fibre protein(s) *in trans* under the control of a suitable promoter. Suitable promoters for the tail fibre hydrid gene(s) may be a phage promoter, particularly the promoter which drives expression of the tail fibre gene in the engineered, obligately-lytic phage. Other suitable promoters are inducible promoters, such as *lac*, and *trp*, together with their cognate regulatory proteins. The SASPject progeny obtained from such strains are extra-potent, carrying the tail fibre hybrid(s) expressed from the strain *in trans* as well as their own. Alternatively, the tail fibre gene from the obligately lytic phage may be deleted altogether, providing that a strain is used for propagation in which tail fibre gene(s) or tail fibre hybrid gene(s) are expressed *in trans*, allowing for the formation of derivative SASPjects. In such an instance, the SASPject progeny from such a strain would carry multiple tail fibres, yet would lack in their genomes any tail fibre or tail fibre hybrid gene(s).

In a further aspect, the present invention provides a composition for inhibiting or preventing bacterial cell growth, which comprises a modified bacteriophage or mixtures thereof as defined herein and a carrier therefor. The modified bacteriophage may be provided in admixture with at least one other modified bacteriophage which is capable of infecting target bacteria, which includes a toxin gene such as a SASP gene encoding a SASP which is toxic to the target bacteria. Then at least one other modified bacteriophage may or may not express a plurality of different HRDs. Such compositions may have a wide range of uses and are therefore to be formulated according to the intended use. The composition may be formulated as a medicament, especially for human treatment and may treat various conditions, including bacterial infections. Among those infections treatable according to the present invention are localised tissue and organ infections, or multi-organ infections, including blood-stream infections, topical infections, oral infections including dental carries, respiratory infections and eye infections. The carrier may be a pharmaceutically-acceptable excipient or diluent. The exact nature and quantities of the components of such compositions may be determined empirically and will depend in part upon the routes of administration of the composition.

Routes of administration to recipients include intravenous, intra-arterial, oral, buccal, sublingual, intranasal, by inhalation, topical (including ophthalmic), intra-muscular, subcutaneous, intra-vaginal, intrathecal and intra-articular. For convenience of use, dosages

according to the invention will depend on the site and type of infection to be treated or prevented. Respiratory infections may be treated by inhalation administration and eye infections may be treated using eye drops. Oral hygiene products containing the modified bacteriophage are also provided; a mouthwash or toothpaste may be used which contains modified bacteriophage according to the invention formulated to eliminate bacteria associated with dental plaque formation.

A modified bacteriophage, or mixture thereof, according to the invention may be used as a bacterial decontaminant, for example in the treatment of surface bacterial contamination as well as land remediation or water treatment. The bacteriophage may be used in the treatment of medical personnel and/or patients as a decontaminating agent, for example in a handwash. Treatment of work surfaces and equipment is also provided, especially that used in hospital procedures or in food preparation. One particular embodiment comprises a composition formulated for topical use for preventing, eliminating or reducing carriage of bacteria and contamination from one individual to another. This is important to limit the transmission of microbial infections, particularly in a hospital environment where bacteria resistant to conventional antibiotics are prevalent. For such a use the modified bacteriophage may be contained in Tris buffered saline or phosphate buffered saline or may be formulated within a gel or cream. For multiple use a preservative may be added. Alternatively the product may be lyophilised and excipients, for example a sugar such as sucrose, may be added.

Detailed description of the invention

This invention will now be described in more detail, by way of example only, and with reference to the accompanying drawings, in which:

Figure 1 is a schematic diagram showing construction of a plasmid containing *lacZ*ΔM15

Figure 2 is a schematic diagram showing construction of plasmids with replaced tail fibre sections;

Figure 3 is a schematic diagram showing construction of phage with hybrid tail fibre genes, which may be subsequently modified to introduce SASP-C according to the invention;

Figure 4 is a schematic diagram showing construction of phage with further hybrid tail fibre genes, which may be subsequently modified to introduce SASP-C according to the invention;

Figure 5 is a schematic diagram showing construction of bacteriophage with hybrid tail fibre genes, in which the *lacZα* marker has been removed;

Figure 6 is a schematic diagram showing construction of plasmids in which SASP-C is introduced into a suitable Phi33 insertion site;

Figure 7 is a schematic diagram showing production of further bacteriophage according to the invention;

Figure 8 is a schematic diagram showing construction of plasmids in which SASP-C codon optimised for expression in *P. aeruginosa* is introduced into a suitable Phi33 insertion site;

Figure 9 shows the sequence of the SASP-C gene from *Bacillus megaterium* strain KM (ATCC 13632), which has been codon optimised for expression in *P. aeruginosa*;

Figure 10 is a schematic diagram showing production of bacteriophage in which SASP-C which has been codon optimised for expression in *P. aeruginosa* is introduced into a suitable Phi33 insertion site;

Figure 11 is a schematic diagram showing production of further bacteriophage according to the invention;

Figure 12 is a schematic diagram showing production of further bacteriophage according to the invention;

Figure 13 is a CLUSTAL 2.1 multiple sequence alignment of the tail fibre proteins from Phage SPM-1, F8, PB1, C36, LBL3, Phi33, LMA2, KPP12, JG024, PTP92, NH-4, 14-1, PTP47, SN; and

Figure 14. 24 hour time-kill curve showing the in vitro efficacy of PT3.9 against *P. aeruginosa* strains 3503 (A) and ATCC 27853 (B). Cultures were grown in Luria bertani (LB) broth

supplemented with 10 mM calcium chloride, 1 mM magnesium sulphate and 1% glucose, at 37 °C.

Figure 15. In vivo efficacy of PT3.9 in a murine bacteraemia model of infection. Mice were treated IV with vehicle (tris buffered saline containing 1 mM magnesium sulphate, 10 mM calcium chloride and 10% v/v glycerol), PTP284 or PTP387 (2 hours post infection with *P. aeruginosa*). The viable cell counts in liver tissue at 22 hours post treatment are shown for each animal in each group (group size = 6), the geomean for each data set is represented by a horizontal line.

Figure 16. In vivo efficacy of PT3.9 in a neutropenic murine pneumonia model of infection. Mice were treated IT (intra-trachea) with placebo vehicle (tris buffered saline containing 1 mM magnesium sulphate, 10 mM calcium chloride and 10% v/v glycerol), PT3.9, PT3.8 or Tobramycin (15 minutes post infection with *P. aeruginosa*). The viable cell counts in lung tissue at 24 hours post treatment are shown for each animal in each group (group size = 6), the geomean for each data set is represented by a horizontal line.

The product of the invention provides in one aspect a single tail fibre within an individual phage, or a mix of phages where each type of phage has a single, different tail fibre.

This is a summary of the genetic modification of a lytic bacteriophage to render it non-lytic, such that it carries one of a number of possible tail fibre variants, in addition to SASP-C under the control of a promoter that usually controls expression of the *Pseudomonas aeruginosa* 30S ribosomal subunit protein S2 gene (*rpsB*), or SASP-C codon optimised for expression in *P. aeruginosa*, under the control of a promoter that usually controls expression of the *P. aeruginosa* fructose-1,6-bisphosphate aldolase gene (*fda*).

Genes can be removed and added to the phage genome using homologous recombination. There are several ways in which phages carrying foreign genes and promoters can be constructed and the following is an example of such methods.

For the construction of a Phi33 derivative it is shown how, using an *E. coli/P. aeruginosa* broad host range vector, as an example only, the existing tail fibre, or a section of the tail fibre, in the

bacteriophage genome may be replaced by an alternative tail fibre or tail fibre section from a different bacteriophage, via homologous recombination. It is also shown as an example only, how additional DNA sequences, such as the SASP-C gene from *B. megaterium* under the control of a *P. aeruginosa rpsB* promoter, or the SASP-C gene from *B. megaterium*, codon optimised for expression in *P. aeruginosa*, under the control of a *P. aeruginosa fda* promoter may be added to the bacteriophage genome via homologous recombination.

A tail fibre gene, or section of a tail fibre gene, from an alternative phage may be cloned between two regions of Phi33 DNA that flank the native tail fibre, or section thereof, along with a *lacZα* genetic marker, in a broad host range *E. coli/P. aeruginosa* vector. This plasmid may be introduced into *P. aeruginosa*, and the resulting strain infected with Phi33. Following harvesting of progeny phage, double recombinants in which the native Phi33 tail fibre or tail fibre section, has been replaced by the new tail fibre or tail fibre section and *lacZα*, may be isolated by plaquing on a suitable *P. aeruginosa* (*lacZ*ΔM15+) host strain using medium containing a chromogenic substrate that detects the action of β-galactosidase.

In a subsequent step, the *lacZα* marker may be removed from the bacteriophage genomes by making versions of the previously described tail fibre region recombination plasmids that do not contain the *lacZα* marker, introducing the new plasmids into suitable *P. aeruginosa* host strains and infecting with the previously modified bacteriophage derivatives of Phi33 carrying the corresponding alternative tail fibre gene, or section thereof, along with the *lacZα* marker. Recombinants that retain the new tail fibre or tail fibre section, but from which *lacZα* has been removed, may be isolated by plaquing on a suitable *P. aeruginosa* (*lacZ*ΔM15+) host strain using medium containing a chromogenic substrate that detects the action of β-galactosidase.

In a subsequent step, a similar homologous recombination may be used to introduce the gene for SASP-C, under the control of a *P. aeruginosa rpsB* promoter, while simultaneously adding an *E. coli lacZα* reporter gene for the identification of recombinant phage, into Phi33, or any of the previously described Phi33 derivatives, or similar bacteriophage or similar derivatives. A region consisting of SASP-C controlled by the *rpsB* promoter, and the *E. coli lacZα* allele, may be cloned between two regions of Phi33 that flank a suitable insertion site, such as the intergenic region located immediately downstream of the Phi33 tail fibre operon, in a broad host range *E. coli/P. aeruginosa* vector. This plasmid may be transferred to a suitable *P. aeruginosa* (*lacZ*ΔM15+) strain, and the resulting strain infected by Phi33 or the previously

constructed Phi33 derivative (from which the initial *lacZα* marker has been removed). Progeny phage may be harvested and double recombinants identified by plaquing on *P. aeruginosa* (*lacZ*ΔM15+), looking for acquisition of the new *lacZα* reporter on medium containing a chromogenic substrate that detects the action of β-galactosidase.

In an alternative subsequent step, a similar homologous recombination may be used to introduce the gene for SASP-C that has been codon optimised for expression in *P. aeruginosa*, under the control of a *P. aeruginosa fda* promoter, while simultaneously adding an *E. coli lacZα* reporter gene for the identification of recombinant phage, into Phi33, or any of the previously described Phi33 derivatives, or similar bacteriophage or similar derivatives. A region consisting of codon optimised SASP-C controlled by the *fda* promoter, and the *E. coli lacZα* allele, may be cloned between two regions of Phi33 that flank a suitable insertion site, such as the intergenic region located immediately downstream of the Phi33 tail fibre operon, in a broad host range *E. coli/P. aeruginosa* vector. This plasmid may be transferred to a suitable *P. aeruginosa* (*lacZ*ΔM15+) strain, and the resulting strain infected by Phi33 or the previously constructed Phi33 derivative (from which the initial *lacZα* marker has been removed). Progeny phage may be harvested and double recombinants identified by plaquing on *P. aeruginosa* (*lacZ*ΔM15+), looking for acquisition of the new *lacZα* reporter on medium containing a chromogenic substrate that detects the action of β-galactosidase.

Since these bacteriophage to be modified are lytic (rather than temperate), another requirement for these described steps of bacteriophage construction is the construction of a suitable host *P. aeruginosa* strain that carries the *E. coli lacZ*ΔM15 gene at a suitable location in the bacterial genome, to complement the *lacZα* phenotypes of the desired recombinant bacteriophage. As an example, the construction of these *P. aeruginosa* strains may be achieved via homologous recombination using an *E. coli* vector that is unable to replicate in *P. aeruginosa*. The genomic location for insertion of the *lacZ*ΔM15 transgene should be chosen such that no essential genes are affected and no unwanted phenotypes are generated through polar effects on the expression of adjacent genes. As an example, one such location may be immediately downstream of the *P. aeruginosa* strain PAO1 *phoA* homologue.

The *E. coli lacZ*ΔM15 allele may be cloned into an *E. coli* vector that is unable to replicate in *P. aeruginosa*, between two regions of *P. aeruginosa* strain PAO1 genomic DNA that flank the 3' end of *phoA*. This plasmid may be introduced into *P. aeruginosa* and isolates having

undergone a single homologous recombination to integrate the whole plasmid into the genome selected according to the acquisition of tetracycline (50 μg/ml) resistance. Isolates (*lacZ*ΔM15+) which have undergone a second homologous recombination event may then be isolated on medium containing 10% sucrose (utilising the *sacB* counter-selectable marker present on the plasmid backbone).

In a subsequent step, a similar homologous recombination may be used to remove the *lacZα* marker from the previously described, (*lacZα*+) Phi33 derivatives that have been modified to introduce the gene for SASP-C, under the control of a *P. aeruginosa rpsB* promoter. A region consisting of SASP-C controlled by the *rpsB* promoter, may be cloned between two regions of Phi33 that flank the chosen insertion site, in a broad host range *E. coli/P. aeruginosa* vector. This plasmid may be transferred to a suitable *P. aeruginosa* (*lacZ*ΔM15+) strain, and the resulting strain infected by the previously described (*lacZα*+) Phi33 derivatives that have been modified to introduce the gene for SASP-C, under the control of a *P. aeruginosa rpsB* promoter. Progeny phage may be harvested and double recombinants identified by plaquing on *P. aeruginosa* (*lacZ*ΔM15+), looking for loss of the *lacZα* reporter on medium containing a chromogenic substrate that detects the action of β-galactosidase.

In an alternative subsequent step, a similar homologous recombination may be used to remove the *lacZα* marker from the previously described, (*lacZα*+) Phi33 derivatives that have been modified to introduce the gene for SASP-C, codon optimised for expression in *P. aeruginosa*, under the control of a *P. aeruginosa fda* promoter. A region consisting of SASP-C, codon optimised for expression in *P. aeruginosa*, controlled by the *fda* promoter, may be cloned between two regions of Phi33 that flank the chosen insertion site, in a broad host range *E. coli/P. aeruginosa* vector. This plasmid may be transferred to a suitable *P. aeruginosa* (*lacZ*ΔM15+) strain, and the resulting strain infected by the previously described (*lacZα*+) Phi33 derivatives that have been modified to introduce the gene for SASP-C, codon optimised for expression in *P. aeruginosa*, under the control of a *P. aeruginosa fda* promoter. Progeny phage may be harvested and double recombinants identified by plaquing on *P. aeruginosa* (*lacZ*ΔM15+), looking for loss of the *lacZα* reporter on medium containing a chromogenic substrate that detects the action of β-galactosidase.

Experimental procedures

PCR reactions to generate DNA for cloning purposes may be carried out using Herculase II Fusion DNA polymerase (Agilent Technologies), depending upon the melting temperatures (Tm) of the primers, according to manufacturers instructions. PCR reactions for screening purposes may be carried out using Taq DNA polymerase (NEB), depending upon the Tm of the primers, according to manufacturers instructions. Unless otherwise stated, general molecular biology techniques, such as restriction enzyme digestion, agarose gel electrophoresis, T4 DNA ligase-dependent ligations, competent cell preparation and transformation may be based upon methods described in Sambrook et al., (1989). Enzymes may be purchased from New England Biolabs or Thermo Scientific. DNA may be purified from enzyme reactions and prepared from cells using Qiagen DNA purification kits. Plasmids may be transferred from *E. coli* strains to *P. aeruginosa* strains by conjugation, mediated by the conjugation helper strain *E. coli* HB101 (pRK2013). A chromogenic substrate for β-galactosidase, S-gal, that upon digestion by β-galactosidase forms a black precipitate when chelated with ferric iron, may be purchased from Sigma (S9811).

Primers may be obtained from Sigma Life Science. Where primers include recognition sequences for restriction enzymes, additional 2-6 nucleotides may be added at the 5' end to ensure digestion of the PCR-amplified DNA.

All clonings, unless otherwise stated, may be achieved by ligating DNAs overnight with T4 DNA ligase and then transforming them into *E. coli* cloning strains, such as DH5α or TOP10, with isolation on selective medium, as described elsewhere (Sambrook et al., 1989).

An *E. coli/P. aeruginosa* broad host range vector, such as pSM1080A, may be used to transfer genes between *E. coli and P. aeruginosa*. pSM1080A was previously produced by combining the broad host-range origin of replication from a *P. aeruginosa* plasmid, *oriT* from pRK2, the *tetAR* selectable marker for use in both *E. coli* and *P. aeruginosa*, from plasmid pRK415, and the high-copy-number, *E. coli* origin of replication, *oriV*, from plasmid pUC19.

An *E. coli* vector that is unable to replicate in *P. aeruginosa*, pSM1104, may be used to generate *P. aeruginosa* mutants by allelic exchange. pSM1104 was previously produced by combining *oriT* from pRK2, the *tetAR* selectable marker for use in both *E. coli* and *P. aeruginosa*, from plasmid pRK415, the high-copy-number, *E. coli* origin of replication, *oriV*, from plasmid

pUC19, and the *sacB* gene from *Bacillus subtilis* strain 168, under the control of a strong promoter, for use as a counter-selectable marker.

Detection of Phi33-like phage (PB1-like phage family) conserved N-terminal tail fibre regions by PCR

1. Primers for the detection of Phi33-like phage-like tail fibre genes in experimental phage samples may be designed as follows:

The DNA sequences of the tail fibre genes from all sequenced Phi33-like phage (including Phi33, PB1, NH-4, 14-1, LMA2, KPP12, JG024, F8, SPM-1, LBL3, PTP47, C36, PTP92 and SN) may be aligned using Clustal Omega, which is available on the EBI website, and the approximately 2 kb-long highly conserved region mapping to the gene's 5' sequence may be thus identified (positions 31680-33557 in the PB1 genome sequence, Acc. EU716414). Sections of 100% identity among the 11 tail fibre gene sequences may be identified by visual inspection. Three pairs of PCR primers targeting selected absolutely conserved regions, and amplifying PCR products no longer than 1 kb may be chosen as follows: pair B4500 and B4501, defining a 193 bp-long region; pair B4502 and B4503, defining a 774 bp-long region; and pair B4504 and B4505, defining a 365 bp-long region.

Primer B4500 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 31680 to 31697. Primer B4501 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 31851 to 31872. Primer B4502 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 31785 to 31804. Primer B4503 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 32541 to 32558. Primer B4504 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 32868 to 32888. Primer B4505 consists of sequence of PB1 phage genome (Acc. EU716414) ranging from position 33213 to 33232.

B4500 (SEQ ID NO: 1)
5'-GTGATCACACCCGAACTG-3'

B4501 (SEQ ID NO: 2)
5'-CGATGAAGAAGAGTTGGTTTTG-3'

B4502 (SEQ ID NO: 3)
5'-ACGCCGGACTACGAAATCAG-3'

B4503 (SEQ ID NO: 4)
5'-TCCGGAGACGTTGATGGT-3'

B4504 (SEQ ID NO: 5)
5'-CCTTTCATCGATTTCCACTTC-3'

B4505 (SEQ ID NO: 6)
5'-TTCGTGGACGCCCAGTCCCA-3'

2. Phi33-like tail fibre genes may be detected in experimental phage samples as follows:

Plaques of isolated phage of environmental origin may be picked from agar plates and added to water and incubated for 30 minutes, making plaque soak outs. The plaque soak outs may be diluted and a portion added to PCR reactions containing one or all of the above primer pairs, and PCR may be performed according to a standard protocol. PCR products may be visualised on a 1.5 % agarose gel with ethidium bromide staining, and evaluated for their size. PCR products of the correct size for the primer pair used may be gel-extracted and submitted to an external facility for sequencing. Sequencing results may be compared with the available tail fibre gene sequences in order to confirm the identity of the PCR product.

Construction of plasmids to generate *Pseudomonas aeruginosa* strains carrying the *Escherichia coli lacZ*ΔM15 gene, immediately downstream of the *phoA* locus of the bacterial genome

1. Plasmid pSMX200 (Figure 1), comprising pSM1104 carrying DNA flanking the 3' end of the *P. aeruginosa* PAO1 *phoA* homologue, may be constructed as follows.

A region comprising the terminal approximately 1 kb of the *phoA* gene from *P. aeruginosa* may be amplified by PCR using primers B4200 and B4201 (Figure 1). The PCR product may then be cleaned and digested with SpeI and BglII. A second region comprising approximately 1 kb downstream of the *phoA* gene from *P. aeruginosa*, including the 3' end of the PA3297

open reading frame, may be amplified by PCR using primers B4202 and B4203 (Figure 1).This second PCR product may then be cleaned and digested with BglII and XhoI. The two digests may be cleaned again and ligated to pSM1104 that has been digested with SpeI and XhoI, in a 3-way ligation, to yield plasmid pSMX200 (Figure 1).

Primer B4200 consists of a 5' SpeI restriction site (underlined), followed by sequence located approximately 1 kb upstream of the stop codon of *phoA* from *P. aeruginosa* strain PAO1 (Figure 1). Primer B4201 consists of 5' BglII and AflII restriction sites (underlined), followed by sequence complementary to the end of the *phoA* gene from *P. aeruginosa* strain PAO1 (the stop codon is in lower case; Figure 1). Primer B4202 consists of 5' BglII and NheI restriction sites (underlined), followed by sequence immediately downstream of the stop codon of the *phoA* gene from *P. aeruginosa* strain PAO1 (Figure 1). Primer B4203 consists of a 5' XhoI restriction site (underlined), followed by sequence within the PA3297 open reading frame, approximately 1 kb downstream of the *phoA* gene from *P. aeruginosa* strain PAO1 (Figure 1).

Primer B4200 (SEQ ID NO:7)
5'-gataACTAGTCCTGGTCCACCGGGGTCAAG-3'

Primer B4201 (SEQ ID NO: 8)
5'-gctcagatcttccttaagtcaGTCGCGCAGGTTCAG-3'

Primer B4202 (SEQ ID NO: 9)
5'-aggaagatctgagctagcTCGGACCAGAACGAAAAAG-3'

Primer B4203 (SEQ ID NO: 10)
5'-gataCTCGAGGCGGATGAACATTGAGGTG-3'

2. Plasmid pSMX203 (Figure 1), comprising pSMX200 carrying *lacZ*ΔM15 under the control of a *lac* promoter, may be constructed as follows.

The *lacZ*ΔM15 gene under the control of a *lac* promoter may be amplified by PCR from *Escherichia coli* strain DH10B using primers B4208 and B4209 (Figure 1). The resulting PCR product may then be digested with BglII and NheI, and ligated to pSMX200 that has also been digested with BglII and NheI, to yield plasmid pSMX203 (Figure 1).

Primer B4208 consists of a 5' BglII restriction site (underlined), followed by sequence of the *lac* promoter (Figure 1). Primer B4209 consists of a 5' NheI restriction site (underlined), followed by a bi-directional transcriptional terminator and sequence complementary to the 3' end of *lacZ*ΔM15 (underlined, in bold; Figure 1).

Primer B4208 (SEQ ID NO: 11)
5'-gataagatctgagcgcaacgcaattaatgtg-3'

Primer B4209 (SEQ ID NO: 12)
5'-gatagctagcAGTCAAAAGCCTCCGGTCGGAGGCTTTTGACTTTATT
TTTGACACCAGACCAAC-3'

Genetic modification of *Pseudomonas aeruginosa* to introduce the *Escherichia coli lacZ*ΔM15 gene immediately downstream of the *phoA* locus of the bacterial genome

1. Plasmid pSMX203 (Figure 1) may be transferred to *P. aeruginosa* by conjugation, selecting for primary recombinants by acquisition of resistance to tetracycline (50 μg/ml).

2. Double recombinants may then be selected via *sacB*-mediated counterselection, by plating onto medium containing 10% sucrose.

3. Isolates growing on 10% sucrose may then be screened by PCR to confirm that *lacZ*ΔM15 has been introduced downstream of the *P. aeruginosa phoA* gene.

4. Following verification of an isolate (PAX21), this strain may then be used as a host for further modification of bacteriophage, where complementation of a *lacZα* reporter is required.

Construction of a plasmid to replace the 3' section of the Phi33 tail fibre with that of PTP92, utilising a *lacZα* screening process

1. pSMX284 (Figure 2), comprising pSM1080A carrying the region immediately downstream of the Phi33 tail fibre gene, may be constructed as follows.

A 1 kb region of Phi33 sequence covering the terminal 20 bases of the Phi33 tail fibre, and the adjacent downstream region, may be amplified by PCR using primers B4222 and B4249 (Figure 2). The resulting PCR product may then be cleaned and digested with NheI, and ligated to pSM1080A that has also been digested with NheI and then treated with alkaline phosphatase prior to ligation, yielding plasmid pSMX284 (Figure 2).

Primer B4222 consists of a 5' NheI restriction site (underlined), followed by sequence from Phi33, approximately 1 kb downstream of the end of the Phi33 tail fibre gene (Figure 2). B4249 consists of 5' NheI-KpnI-AvrII restriction sites (underlined), followed by sequence complementary to the 3' end of the Phi33 tail fibre and sequence immediately downstream of the tail fibre open reading frame (Figure 2).

B4222 (SEQ ID NO: 13)
5'-gataGCTAGCATGGTTTTCACGACCATG-3'

B4249 (SEQ ID NO: 14)
5'-GATAGCTAGCGAGGTACCGACCTAGGTTTTCCAGCGAGTGACGTAA
AATG-3'

2. pSMX285 (Figure 2), comprising pSMX284 carrying *lacZα*, a 3' section of the PTP92 tail fibre gene sequence, and a region of Phi33 sequence comprising the 5' end of the tail fibre gene and sequence located immediately upstream of the Phi33 tail fibre gene, may be constructed as follows.

The *lacZα* open reading frame may be amplified by PCR from pUC19 using primers B4250 and B4252 (Figure 2). The PTP92 tail fibre 3' section may be amplified by PCR from PTP92 using primers B4251 and B4254 (Figure 2). The *lacZα* open reading frame may then be joined to the PTP92 tail fibre gene 3' section by SOEing PCR using the outer primers, B4250 and B4254. A region comprising sequence of the 5' end of the Phi33 tail fibre gene, and sequence located immediately upstream of the Phi33 tail fibre gene, may be amplified by PCR using primers B4253 and B4229 (Figure 2). This PCR product may then be joined to the PCR product comprising *lacZα* and the PTP92 tail fibre gene 3' section, by SOEing PCR using the outer primers B4250 and B4229. The resulting PCR product may then be cleaned and digested with

AvrII and KpnI, and ligated to pSMX284 that has also been digested with AvrII and KpnI, yielding plasmid pSMX285 (Figure 2).

Primer B4250 consists of a 5' AvrII restriction site, followed by sequence complementary to the 3' end of the *lacZα* open reading frame (Figure 2). Primer B4252 consists of a 5' section of sequence that overlaps the 3' end of the PTP92 tail fibre gene (underlined), followed by sequence of the 5' end of the *lacZα* open reading frame. Primer B4251 is the reverse complement of primer B4252 (Figure 2). Primer B4254 consists of 5' sequence from within the Phi33 tail fibre gene (underlined), followed by sequence within the 3' end of the PTP92 tail fibre gene (Figure 2). Primer B4253 is the reverse complement of Primer B4254. Primer B4229 consists of a 5' KpnI restriction site (underlined), followed by sequence that is complementary to a region approximately 1 kb upstream of the tail fibre gene in Phi33 (Figure 2).

Primer B4250 (SEQ ID NO: 15)
5'-GataCCTAGGttagcgccattcgccattc-3'

Primer B4252 (SEQ ID NO: 16)
5'-CTATTCCAGCGGGTAACGTAAAatgaccatgattacggattC-3'

Primer B4251 (SEQ ID NO: 17)
5'-GaatccgtaatcatggtcatTTTACGTTACCCGCTGGAATAG-3'

Primer B4254 (SEQ ID NO: 18)
5'-CAAGCGGGCCGGCTGGTCTCTCGGCAATAACTCCTATGTGATC-3'

Primer B4253 (SEQ ID NO: 19)
5'-GATCACATAGGAGTTATTGCCGAGAGACCAGCCGGCCCGCTTG-3'

Primer B4229 (SEQ ID NO: 20)
5'-gataGGTACCGCGACCGGTCTGTACTTC-3'

Genetic modification of Phi33 to replace the 3' section of the tail fibre gene with that of PTP92

1. Plasmid pSMX285 (Figure 2; Figure 3) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA80.

2. Strain PTA80 may be infected with phage Phi33, and the progeny phage harvested.

3. Recombinant phage in which the 3' end of the Phi33 tail fibre gene has been replaced by that of PTP92, and to which *lacZα* has been added, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for black plaques, which are indicative of β-galactosidase activity.

4. PCR may be carried out to check that the tail fibre gene has been replaced, and that *lacZα* is present.

5. Following identification of a verified isolate (PTPX81; Figure 3), this isolate may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Construction of a plasmid to replace the 3' section of the Phi33 tail fibre with that of PTP47, utilising a *lacZα* screening process

1. pSMX286 (Figure 2), comprising pSMX284 carrying *lacZα*, a 3' section of the PTP47 tail fibre gene sequence, and a region of Phi33 sequence comprising the 5' end of the tail fibre gene and sequence located immediately upstream of the Phi33 tail fibre gene, may be constructed as follows.

The *lacZα* open reading frame may be amplified by PCR from pUC19 using primers B4250 and B4258 (Figure 2). The PTP47 tail fibre 3' section may be amplified by PCR from PTP47 using primers B4259 and B4260 (Figure 2). The *lacZα* open reading frame may then be joined to the PTP47 tail fibre gene 3' section by SOEing PCR using the outer primers, B4250 and B4260. A region comprising sequence of the 5' end of the Phi33 tail fibre gene, and sequence located immediately upstream of the Phi33 tail fibre gene, may be amplified by PCR using primers B4261 and B4229 (Figure 2). This PCR product may then be joined to the PCR product comprising *lacZα* and the PTP47 tail fibre gene 3' section, by SOEing PCR using the outer primers B4250 and B4229. The resulting PCR product may then be cleaned and digested with

AvrII and KpnI, and ligated to pSMX284 that has also been digested with AvrII and KpnI, yielding plasmid pSMX286 (Figure 2).

Primer B4250 consists of a 5' AvrII restriction site, followed by sequence complementary to the 3' end of the *lacZα* open reading frame (Figure 2). Primer B4258 consists of a 5' section of sequence that overlaps the 3' end of the PTP47 tail fibre gene (underlined), followed by sequence of the 5' end of the *lacZα* open reading frame. Primer B4259 is the reverse complement of primer B4258 (Figure 2). Primer B4260 consists of 5' sequence from within the Phi33 tail fibre gene (underlined), followed by sequence within the 3' end of the PTP47 tail fibre gene (Figure 2). Primer B4261 is the reverse complement of Primer B4260. Primer B4229 consists of a 5' KpnI restriction site (underlined), followed by sequence that is complementary to a region approximately 1 kb upstream of the tail fibre gene in Phi33 (Figure 2).

Primer B4250 (SEQ ID NO: 15)
5'-GataCCTAGGttagcgccattcgccattc-3'

Primer B4258 (SEQ ID NO: 21)
5'-CTTTTCCAGCGAGTGACGTAAAatgaccatgattacggattC-3'

Primer B4259 (SEQ ID NO: 22)
5'-gaatccgtaatcatggtcatTTTACGTCACTCGCTGGAAAAG-3'

Primer B4260 (SEQ ID NO: 23)
5'-CAAGCGGGCCGGCTGGTCTCTCGGCAATAACTCCTATGTGATC-3'

Primer B4261 (SEQ ID NO: 24)
5'-GATCACATAGGAGTTATTGCCGAGAGACCAGCCGGCCCGCTTG-3'

Primer B4229 (SEQ ID NO: 20)
5'-gataGGTACCGCGACCGGTCTGTACTTC-3'

Genetic modification of Phi33 to replace the 3' section of the tail fibre gene with that of PTP47

1. Plasmid pSMX286 (Figure 2; Figure 4) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA81.

2. Strain PTA81 may be infected with phage Phi33, and the progeny phage harvested.

3. Recombinant phage in which the 3' end of the Phi33 tail fibre gene has been replaced by that of PTP47, and to which *lacZα* has been added, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for black plaques, which are indicative of β-galactosidase activity.

4. PCR may be carried out to check that the tail fibre gene has been replaced, and that *lacZα* is present.

5. Following identification of a verified isolate (PTPX82; Figure 4), this isolate may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Construction of a plasmid to remove the *lacZα* marker from PTPX81

1. pSMX287 (Figure 5), comprising pSM1080A carrying a 3' section of the PTP92 tail fibre gene, and a region of Phi33 sequence located immediately downstream of the Phi33 tail fibre gene, may be constructed as follows.

The region of Phi33 sequence located immediately downstream of the Phi33 tail fibre may be amplified by PCR using primers B4222 and B4255 (Figure 5). The 3' end of the PTP92 tail fibre gene may be amplified by PCR using primers B4256 and B4257 (Figure 5). These two PCR products may then be joined by SOEing PCR, using the two outer primers B4222 and B4257. The resulting PCR product may then be cleaned, digested with NheI, cleaned again, and ligated to pSM1080A that has also been digested with NheI and then treated with alkaline phosphatase prior to ligation, to yield plasmid pSMX287 (Figure 5).

Primer B4255 consists of a 5' section of the end of the PTP92 tail fibre gene (underlined), followed by sequence immediately downstream of the Phi33 tail fibre gene (Figure 5). Primer B4256 is the reverse complement of primer B4255 (Figure 5). Primer B4257 consists of a 5'

NheI restriction site (underlined), followed by sequence of the terminal 1 kb of the PTP92 tail fibre gene (Figure 5).

Primer B4255 (SEQ ID NO: 25)
5'-CTATTCCAGCGGGTAACGTAAAATGAAATGGACGCGGATCAG-3'

Primer B4256 (SEQ ID NO: 26)
5'-CTGATCCGCGTCCATTTCATTTTACGTTACCCGCTGGAATAG-3'

Primers B4257 (SEQ ID NO: 27)
5'-gataGCTAGCGGCAATAACTCCTATGTGATC-3'

Genetic modification of PTPX81 to remove the *lacZα* marker

1. Plasmid pSMX287 (Figure 5; Figure 3) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA82.

2. Strain PTA82 may be infected with phage PTPX81, and the progeny phage harvested.

3. Recombinant phage in which the *lacZα* marker has been removed may be identified by plaquing the lysate from step (2) on P. aeruginosa strain PAX21, onto medium containing S-gal, looking for white plaques, which are indicative of loss of β-galactosidase activity.

4. PCR may be carried out to check that the tail fibre gene has been retained, and that *lacZα* has been removed.

5. Following identification of a verified isolate (PTPX83; Figure 3), this isolate may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Construction of a plasmid to remove the *lacZα* marker from PTPX82

1. pSMX288 (Figure 5), comprising pSM1080A carrying a 3' section of the PTP47 tail fibre gene, and a region of Phi33 sequence located immediately downstream of the Phi33 tail fibre gene, may be constructed as follows.

The region of Phi33 sequence located immediately downstream of the Phi33 tail fibre may be amplified by PCR using primers B4222 and B4262 (Figure 5). The 3' end of the PTP47 tail fibre gene may be amplified by PCR using primers B4263 and B4264 (Figure 5). These two PCR products may then be joined by SOEing PCR, using the two outer primers B4222 and B4264. The resulting PCR product may then be cleaned, digested with NheI, cleaned again, and ligated to pSM1080A that has also been digested with NheI and then treated with alkaline phosphatase prior to ligation, to yield plasmid pSMX288 (Figure 5).

Primer B4262 consists of a 5' section of the end of the PTP47 tail fibre gene (underlined), followed by sequence immediately downstream of the Phi33 tail fibre gene (Figure 5). Primer B4263 is the reverse complement of primer B4262 (Figure 5). Primer B4264 consists of a 5' NheI restriction site (underlined), followed by sequence of the terminal 1 kb of the PTP47 tail fibre gene (Figure 5).

Primer B4262 (SEQ ID NO: 28)
5'-CTTTTCCAGCGAGTGACGTAAAATGAAATGGACGCGGATCAG-3'

Primer B4263 (SEQ ID NO: 29)
5'-CTGATCCGCGTCCATTTCATTTTACGTCACTCGCTGGAAAAG -3'

Primers B4264 (SEQ ID NO: 30)
5'-gataGCTAGCGGCAATAACTCCTATGTGATC-3'

Genetic modification of PTPX82 to remove the *lacZα* marker

1. Plasmid pSMX288 (Figure 5; Figure 4) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA83.

2. Strain PTA83 may be infected with phage PTPX82, and the progeny phage harvested.

3. Recombinant phage in which the *lacZα* marker has been removed may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for white plaques, which are indicative of loss of β-galactosidase activity.

4. PCR may be carried out to check that the tail fibre gene has been retained, and that *lacZα* has been removed.

5. Following identification of a verified isolate (PTPX84; Figure 4), this isolate may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Construction of a plasmid to introduce *rpsB*-SASP-C and *lacZα* into the phage genome of Phi33, PTPX83, PTPX84, and similar phage

1. Plasmid pSMX251 (Figure 6), comprising pSM1080A containing regions of Phi33 flanking the chosen insertion site for *rpsB*-SASP-C, such as the intergenic region immediately downstream of the tail fibre operon, may be constructed as follows.

The region of Phi33 sequence immediately downstream of the chosen insertion site may be amplified by PCR using primers B4900 and B4901 (Figure 6). This PCR product may then be cleaned and digested with NheI and AvrII. The region of Phi33 sequence immediately upstream of the chosen insertion site may be amplified by PCR using primers B4902 and B4903 (Figure 6). This second PCR product may then be cleaned and digested with AvrII and NheI. The two PCR product digests may then be cleaned again and ligated to pSM1080A that has been digested with NheI and treated with alkaline phosphatase prior to ligation. Clones carrying one insert of each of the two PCR products may be identified by PCR using primers B4900 and B4903, and NheI restriction digest analysis of the purified putative clones, to identify plasmid pSMX251 (Figure 6).

Primer B4900 consists of a 5' NheI restriction site (underlined), followed by Phi33 sequence located approximately 500bp downstream of the Phi33 insertion site that is within the intergenic region immediately downstream of the tail fibre operon (Figure 6). Primer B4901 consists of 5' AvrII and XhoI restriction sites (underlined), followed by sequence of Phi33 that

is complementary to sequence located immediately downstream of the Phi33 insertion site (Figure 6). Primer B4902 consists of a 5' AvrII restriction site (underlined), followed by Phi33 sequence located immediately upstream of the insertion site (Figure 6). Primer B4903 consists of a 5' NheI site (underlined), followed by Phi33 sequence that is complementary to sequence located approximately 500bp upstream of the Phi33 insertion site (Figure 6).

Primer B4900 (SEQ ID NO: 31)
5' gatagctagcTTTCTCGTTTTAATGTCG 3'

Primer B4901 (SEQ ID NO: 32)
5' gataCCTAGGtgCTCGAGTATTCGCCCAAAAGAAAAG 3'

Primer B4902 (SEQ ID NO: 33)
5' gataCCTAGGTCAGGAGCCTTGATTGATC 3'

Primer B4903 (SEQ ID NO: 34)
5' gatagctagcGGACTGGTAAGTCTGGTG 3'

2. Plasmid pSMX252 (Figure 6), comprising pSMX251 containing SASP-C under the control of an *rpsB* promoter, may be constructed as follows.

The SASP-C gene from *Bacillus megaterium* strain KM (ATCC 13632) may be amplified by PCR using primers B4904 and B4270 (Figure 6). The resulting PCR product may then be digested with XhoI and NcoI. The *rpsB* promoter may be amplified by PCR from *P. aeruginosa* using primers B4271 and B4905 (Figure 6). The resulting PCR product may then be digested with NcoI and AvrII. The two digested PCR products may then be cleaned and ligated to pSMX251 that has been digested with XhoI and AvrII, yielding plasmid pSMX252 (Figure 6).

Primer B4904 comprises a 5' XhoI restriction site, followed by 5 bases, and then a bi-directional transcriptional terminator, and then sequence complementary to the 3' end of the SASP-C gene from *B. megaterium* strain KM (ATCC 13632) (underlined, in bold; Figure 6). Primer B4270 comprises a 5' NcoI restriction site (underlined), followed by sequence of the 5' end of the SASP-C gene from *B. megaterium* strain KM (ATCC 13632) (Figure 6). Primer B4271 comprises a 5' NcoI restriction site (underlined), followed by sequence complementary

to the end of the *rpsB* promoter from *P. aeruginosa* PAO1 (Figure 6). Primer B4905 comprises a 5' AvrII restriction site (underlined), followed by sequence of the beginning of the *rpsB* promoter from *P. aeruginosa* PAO1 (Figure 6).

Primer B4904 (SEQ ID NO: 35)
5'-gataCTCGAGGATCTAGTCAAAAGCCTCCGACCGGAGGCTTTTGACT
ttagtacttgccgcctag-3'

Primer B4270 (SEQ ID NO: 36)
5'- gataccATGGcaaattatcaaaacgcatc-3'

Primer B4271 (SEQ ID NO: 37)
5'-gataCCATggTAGTTCCTCGATAAGTCG-3'

Primer B4905 (SEQ ID NO: 38)
5'-gataCCTAGGCCTAGGgatctGACCGACCGATCTACTCC-3'

3. pSMX253 (Figure 6), comprising pSMX252 containing *lacZα*, may be constructed as follows.

lacZα may be PCR amplified using primers B4906 and B4907 (Figure 6). The resulting PCR product may then be digested with XhoI and ligated to pSMX252 that has also been digested with XhoI and treated with alkaline phosphatase prior to ligation, to yield pSMX253 (Figure 6).

Primer B4906 consists of a 5' XhoI restriction site (underlined), followed by sequence complementary to the 3' end of *lacZα* (Figure 6). Primer B4907 consists of a 5' XhoI restriction site (underlined), followed by sequence of the *lac* promoter driving expression of *lacZα* (Figure 6).

Primer B4906 (SEQ ID NO: 39)
5'-gataCTCGAGttagcgccattcgccattc-3'

Primer B4907 (SEQ ID NO: 40)

5'-gataCTCGAGgcgcaacgcaattaatgtg-3'

Genetic modification of Phi33, PTPX83, PTPX84, and similar phage, to introduce *rpsB*-SASP-C and *lacZα*

1. Plasmid pSMX253 (Figure 6; Figure 3; Figure 4; Figure 7) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA51.

2. Strain PTA51 may be infected in individual experiments with phage Phi33, or PTPX83, or PTPX84, or other similar phage, and the progeny phage harvested.

3. Recombinant phage, in which *rpsB*-SASP-C and *lacZα* have been introduced into the chosen insertion site, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for black plaques, which are indicative of β-galactosidase activity.

4. PCR may be carried out to check that *rpsB*-SASP-C and *lacZα* are present.

5. Following identification of verified isolates (for example, PTPX85 (Figure 7), PTPX86 (Figure 3), PTPX87 (Figure 4)), the isolates may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Genetic modification to remove the *lacZα* marker from PTPX85, PTPX86, PTPX87, and similar derivatives of Phi33

1. Plasmid pSMX252 (Figure 6; Figure 3; Figure 4; Figure 7) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA85.

2. Strain PTA85 may be infected in individual experiments with phage PTPX85, or PTPX86, or PTPX87, or other similar phage, and the progeny phage harvested.

3. Recombinant phage, in which *lacZα* marker has been removed, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for white plaques, which are indicative of loss of β-galactosidase activity.

4. PCR may be carried out to confirm removal of the *lacZα* marker, while ensuring that *rpsB*-SASP-C is still present.

5. Following identification of verified isolates (for example, PTPX88 (Figure 7), PTPX89 (Figure 3), PTPX90 (Figure 4)), the isolates may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Construction of a plasmid to introduce *fda*-SASP-C (codon optimised) and *lacZα* into a chosen insertion site, located in an intergenic region immediately downstream of the tail fibre operon, within the genome of Phi33, PTPX83, PTPX84, and similar phage

1. Plasmid pSMX254 (Figure 8), comprising pSMX251 containing SASP-C codon optimised for expression in *P. aeruginosa*, under the control of an *fda* promoter, may be constructed as follows.

The SASP-C gene from *Bacillus megaterium* strain KM (ATCC 13632) may be codon optimised for expression in *P. aeruginosa* (Figure 9) and synthesised *in vitro*. The codon optimised SASP-C gene may then be amplified by PCR using primers B4312 and B4313 (Figure 8). The *fda* promoter may be amplified by PCR from *P. aeruginosa* using primers B4314 and B4315 (Figure 8). The resulting two PCR products may then be joined by splicing by overlap extension (SOEing) PCR, using the outer primers B4312 and B4314 (Figure 8). The resulting *fda*-codon optimised SASP-C-terminator PCR product may then be digested with XhoI and AvrII, cleaned, and ligated to pSMX251 that has been digested with XhoI and AvrII, yielding plasmid pSMX254 (Figure 8).

Primer B4312 comprises a 5' XhoI restriction site, followed by a bi-directional transcriptional terminator, and then sequence complementary to the 3' end of the SASP-C gene from *B. megaterium* strain KM (ATCC 13632) that has been codon optimised for expression in *P. aeruginosa* (underlined, in bold; Figure 8). Primer B4313 comprises sequence of the 3' end of the *fda* promoter from *P. aeruginosa* PAO1 (in bold) followed by sequence of the 5' end of the codon optimised SASP-C gene. Primer B4314 comprises sequence complementary to the 5'

end of the codon optimised SASP-C gene followed by sequence complementary to the 3' end of the *fda* promoter from *P. aeruginosa* PAO1 (Figure 8). Primer B4315 comprises a 5' AvrII restriction site (underlined), followed by sequence of the beginning of the *fda* promoter from *P. aeruginosa* PAO1 (Figure 8).

Primer B4312 (SEQ ID NO: 41)
5'-
gataCTCGAGAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTTCAGTACTTGCCGC
CCAG-3'

Primer B4313 (SEQ ID NO: 42)
5'- GATTGGGAGATACGAGAACCATGGCCAACTACCAGAACGC -3'

Primer B4314 (SEQ ID NO: 43)
5'- GCGTTCTGGTAGTTGGCCATGGTTCTCGTATCTCCCAATC-3'

Primer B4315 (SEQ ID NO: 44)
5'- GATACCTAGGAACGACGAAGGCCTGGTG-3'

3. pSMX255 (Figure 8), comprising pSMX254 containing *lacZα*, may be constructed as follows.

lacZα may be PCR amplified using primers B4906 and B4907 (Figure 8). The resulting PCR product may then be digested with XhoI and ligated to pSMX254 that has also been digested with XhoI and treated with alkaline phosphatase prior to ligation, to yield pSMX255 (Figure 8).

Primer B4906 consists of a 5' XhoI restriction site (underlined), followed by sequence complementary to the 3' end of *lacZα* (Figure 8). Primer B4907 consists of a 5' XhoI restriction site (underlined), followed by sequence of the *lac* promoter driving expression of *lacZα* (Figure 8).

Primer B4906 (SEQ ID NO: 40)
5'-gataCTCGAGttagcgccattcgccattc-3'

Primer B4907 (SEQ ID NO: 41)
5'-gataCTCGAGgcgcaacgcaattaatgtg-3'

Genetic modification of Phi33, PTPX83, PTPX84, and similar phage, to introduce *fda*-codon optimised SASP-C and *lacZα*

1. Plasmid pSMX255 (Figure 8; Figure 10; Figure 11; Figure 12) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA86.

2. Strain PTA86 may be infected in individual experiments with phage Phi33, or PTPX83, or PTPX84, or other similar phage, and the progeny phage harvested.

3. Recombinant phage, into which *fda*-codon optimised SASP-C and *lacZα* have been introduced, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for black plaques, which are indicative of β-galactosidase activity.

4. PCR may be carried out to check that *fda*-codon optimised SASP-C and *lacZα* are present.

5. Following identification of verified isolates (for example, PTPX91 (Figure 12), PTPX92 (Figure 10), PTPX93 (Figure 11)), the isolates may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

Genetic modification to remove the *lacZα* marker from PTPX91, PTPX92, PTPX93, and similar derivatives of Phi33

1. Plasmid pSMX254 (Figure 8; Figure 10; Figure 11; Figure 12) may be introduced into *P. aeruginosa* strain PAX21 by conjugation, selecting transconjugants on the basis of tetracycline resistance (50 μg/ml), yielding strain PTA87.

2. Strain PTA87 may be infected in individual experiments with phage PTPX91, or PTPX92, or PTPX93, or other similar phage, and the progeny phage harvested.

3. Recombinant phage, in which *lacZα* marker has been removed, may be identified by plaquing the lysate from step (2) on *P. aeruginosa* strain PAX21, onto medium containing S-gal, looking for white plaques, which are indicative of loss of β-galactosidase activity.

4. PCR may be carried out to confirm removal of the *lacZα* marker, while ensuring that *fda*-codon optimised SASP-C is still present.

5. Following identification of verified isolates (for example, PTP387 (Figure 12), PTP388 (Figure 10), PTP389 (Figure 11)), the isolates may be plaque purified twice more on *P. aeruginosa* strain PAX21, prior to further use.

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Table 1. Host range of Phi33, PTP92, C36 and PTP47 against 44 European clinical isolates of *Pseudomonas aeruginosa*.

Strains were tested for sensitivity to each phage by dropping 10 µl of crude phage lysate onto a soft agar overlay plate inoculated with bacteria. Plates were grown overnight at 32°C and the strains were scored for sensitivity to each phage by assessing clearance zones at the point of inoculation. Where phage inhibited growth, as seen by clearance of the bacterial lawn, the strain was marked as sensitive (+), and where no inhibition of growth was seen, the strain was marked as not-sensitive (-)

Bacterial Strain no.	Phi33	PTP47	PTP92	C36
2019	+	+	-	+
2020	+	+	-	+
2021	+	+	+	+
2029	+	+	-	+
2031	+	+	+	+
2039	+	+	+	+
2040	+	+	-	+
2041	+	+	+	+
2042	+	+	+	+
2045	-	-	+	-
2046	+	+	+	+
2047	+	+	+	+
2048	+	+	+	+
2049	+	+	+	+
2050	+	+	+	+
2051	+	+	-	-
2052	-	-	-	-
2053	+	+	-	+
2054	-	+	-	+
2055	+	+	-	+
2056	+	+	+	+
2057	+	+	+	+
2058	+	+	+	+
2483	-	-	+	-
2484	+	+	-	+
2705	+	+	-	+
2706	+	+	-	+
2707	+	+	+	+
2708	+	+	+	+
2709	+	+	+	+
2710	-	+	+	-
2711	+	+	+	+
2712	+	+	-	+
2713	-	+	+	+
2714	+	+	+	+
2715	+	+	+	+
2716	+	+	-	-
2717	-	+	+	+
2718	-	+	+	+
2719	+	+	-	+
2720	+	+	+	+
2721	+	+	+	+
2722	+	+	+	+
2723	+	+	-	+

Table 2. Host range of Phi33, PTP92 and PTP93 against 35 European clinical isolates of *Pseudomonas aeruginosa*. Strains were tested for sensitivity to each phage by dropping 10 µl of crude phage lysate onto a soft agar overlay plate inoculated with bacteria. Plates were grown overnight at 32°C and the strains were scored for sensitivity to each phage by assessing clearance zones at the point of inoculation. Where phage inhibited growth, as seen by clearance of the bacterial lawn, the strain was marked as sensitive (+), and where no inhibition of growth was seen, the strain was marked as not-sensitive (-)

Isolate	Phi33	PTP93	PTP92
2019	+	+	-
2020	+	+	-
2029	+	+	-
2040	+	+	-
2045	-	+	+
2053	+	+	-
2483	-	+	+
2484	+	+	-
2705	+	-	-
2710	-	+	+
2711	+	+	+
2712	+	+	-
2713	-	+	+
2716	+	+	-
2717	-	+	+
2718	-	+	+
2720	+	+	+
2721	+	+	+
2722	+	+	+
2723	+	-	-
2728	-	+	+
2733	+	+	-
2734	+	+	+
2740	-	+	+
2741	+	+	+
2742	+	+	+
2743	+	+	+
2747	+	+	+
2748	+	+	+
2749	+	+	-
2750	+	+	+
2752	+	+	+
2753	-	+	+
2754	+	+	+
2756	+	+	+

Claims:

1. A modified bacteriophage capable of infecting a plurality of different target bacteria, which bacteriophage includes a toxin gene encoding a toxin protein which is toxic to the target bacteria; wherein the bacteriophage is lytic; and wherein the bacteriophage expresses host range determinant proteins which have a plurality of bacterial host specificities.

2. A modified bacteriophage according to claim 1, wherein the toxin gene comprises an α/β small acid-soluble spore protein (SASP) gene encoding a SASP.

3. A modified bacteriophage according to claim 1 or claim 2, wherein the SASP is SASP-C.

4. A modified bacteriophage according to claim 3, wherein the SASP-C is from *Bacillus megaterium*.

5. A modified bacteriophage according to claim 1, wherein the bacterial host specificity of the host range determinant is within the same bacterial species.

6. A modified bacteriophage according to any one of the preceding claims, wherein the toxin gene is under the control of a constitutive promoter.

7. A modified bacteriophage according to claim 6, wherein the constitutive promoter is selected from *pdhA*, *rpsB*, *pgi*, *fda*, *lasB* and promoters having more than 90% sequence identity thereto.

8. A modified bacteriophage according to claim 7, wherein the constitutive promoter is *fda*.

9. A modified bacteriophage according to any one of the preceding claims, wherein at least one of the target bacteria is *Pseudomonas*.

10. A modified bacteriophage according to claim 9, wherein the plurality of different target bacteria is a plurality of different *Pseudomonas* bacteria.

11. A modified bacteriophage according to claim 9 or claim 10, wherein the *Pseudomonas* bacteria comprise *Pseudomonas aeruginosa*.

12. A modified bacteriophage according to any one of the preceding claims, wherein the hybrid host range determinants have a broad host range as defined by more than 50% of a collection of at least 35 and preferably more than 50 clinical isolates, from a plurality of different infection sites and including a range of antibiotic resistance phenotypes.

13. A modified bacteriophage according to any one of the preceding claims, wherein the host range determinants comprise tail fibre proteins.

14. A modified bacteriophage according to claim 13, wherein each tail fibre protein comprises a receptor binding region for binding to the target bacteria and a region linking the receptor binding region to the body of the bacteriophage.

15. A modified bacteriophage according to claim 14, wherein the receptor binding region is a C-terminal receptor binding region and the region linking the C-terminal receptor binding region to the body of the bacteriophage is an N-terminal region.

16. A modified bacteriophage according to claim 15, wherein the N-terminal region comprises amino acids 1 to 628 of the tail fibre protein and the C-terminal region comprises amino acids 629 to 964 of the tail fibre protein, based on the amino acid sequence of bacteriophage Phi33.

17. A modified bacteriophage according to claim 15 or claim 16, wherein the C-terminal region has no more than 96% amino acid sequence identity with the C-terminal region of bacteriophage Phi33.

18. A modified bacteriophage according to claim 17, wherein the C-terminal region is from any one of bacteriophage Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH4, PTP47, PTP92, C36 and PTP93.

19. A modified bacteriophage according to claim 18, wherein the C-terminal region amino sequence identity is less than 80%.

20. A modified bacteriophage according to claim 19, wherein the C-terminal region amino acid sequence identity is less than 70%.

21. A modified bacteriophage according to claim 20, wherein the C-terminal region amino acid sequence identity is less than 60%.

22. A modified bacteriophage according to any one of claims 14 to 21, wherein the N-terminal region has at least 95% amino acid sequence identity with the N-terminal region of bacteriophage Phi33.

23. A modified bacteriophage according to claim 22, wherein the N-terminal region is from any one of bacteriophage Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH4, PTP47, PTP92, C36 and PTP93.

24. A modified bacteriophage according to any one of the preceding claims wherein the host range determinant proteins are hybrid host range determinant proteins each comprising an amino acid sequence from a plurality of different bacteriophages.

25. A modified bacteriophage according to claim 24, wherein the hybrid host range determinant proteins comprise hybrid tail fibre proteins comprising a C-terminal receptor binding region and an N-terminal region linking the C-terminal receptor binding region to the body of the bacteriophage, wherein the C-terminal and N-terminal regions are each from a different bacteriophage.

26. A modified bacteriophage according to claim 25, wherein each hybrid tail fibre protein comprises the C-terminal receptor binding region of bacteriophage PTP47 and the N-terminal region of bacteriophage Phi33.

27. A modified bacteriophage according to claim 25, wherein each hybrid tail fibre protein comprises the C-terminal receptor binding region of bacteriophage PTP92 and the N-terminal region of bacteriophage Phi33.

28. A modified bacteriophage according to any one of claims 1 to 23, wherein the bacteriophage expresses a plurality of different host range determinants; and wherein each host range determinant has a different bacterial host specificity.

29. A modified bacteriophage according to claim 28, wherein each host range determinant protein comprises a tail fibre protein which is from a bacteriophage selected from Phi33, LBL3, SPM-1, F8, PB1, KPP12, LMA2, SN, 14-1, JG024, NH4, PTP47, PTP92, C36 and PTP93.

30. A modified bacteriophage according to claim 23, which is bacteriophage PTP387.

31. A modified bacteriophage according to any one of the preceding claims in admixture with at least one other modified bacteriophage which is capable of infecting target bacteria, which includes a SASP gene encoding a SASP which is toxic to the target bacteria.

32. A modified bacteriophage according to any one of the preceding claims, for use as a medicament.

33. A modified bacteriophage according to any one of the preceding claims, for use in the treatment of bacterial infection.

34. A modified bacteriophage according to claim 33, wherein the bacterial infection comprises a localised organ infection or a multi-organ infection.

35. A modified bacteriophage according to claim 34, where the infection comprises a topical infection, oral infection, respiratory infection, eye infection or blood stream infection.

36. A modified bacteriophage according to any one of claims 32 to 35, which is for human therapy.

37. A modified bacteriophage according to any one of claims 1 to 31, for use in the therapeutic inhibition or prevention of bacterial cell growth.

38. A composition for inhibiting or preventing bacterial cell growth, which comprises a modified bacteriophage according to any one of claims 1 to 31, and a carrier therefor.

39. A composition according to claim 38, which comprises a plurality of the modified bacteriophages, at least two of which have different host specificities.

40. A composition according to claim 38, which comprises bacteriophage PTP387, bacteriophage PTP388 and bacteriophage PTP389.

41. A composition according to any one of claims 38 to 40, which is formulated for pharmaceutical use.

42. A composition according to any one of claims 38 to 41, which is formulated for topical use.

43. A composition according to any one of claims 38 to 41, which is formulated for delivery to the respiratory tract.

44. Use of a modified bacteriophage according to any one of claims 1 to 31, as a bacterial decontaminant.

45. Use according to claim 44, which comprises treating surface bacterial contamination, land remediation or water treatment.

Figure 1 (Part 1 of 2)

Figure 1 (Part 2 of 2)

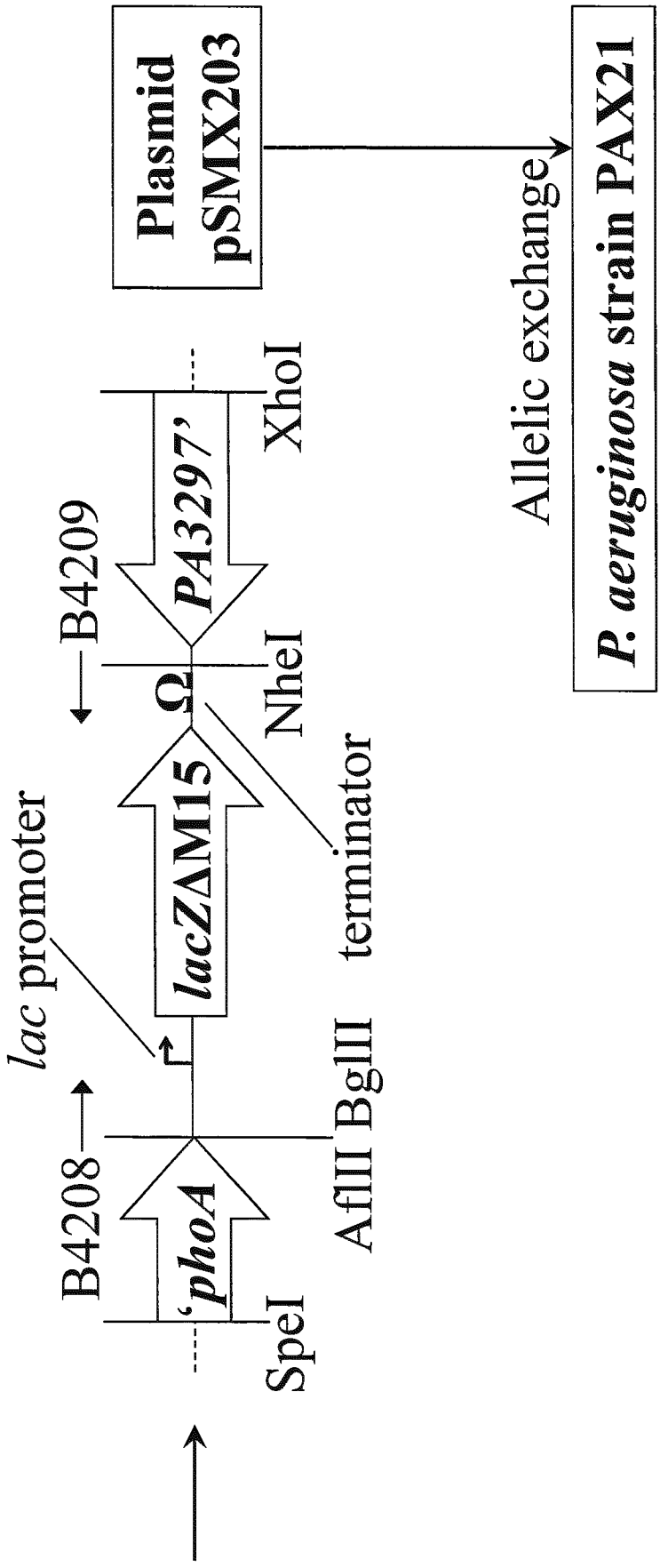


Figure 2 (Part 1 of 4)

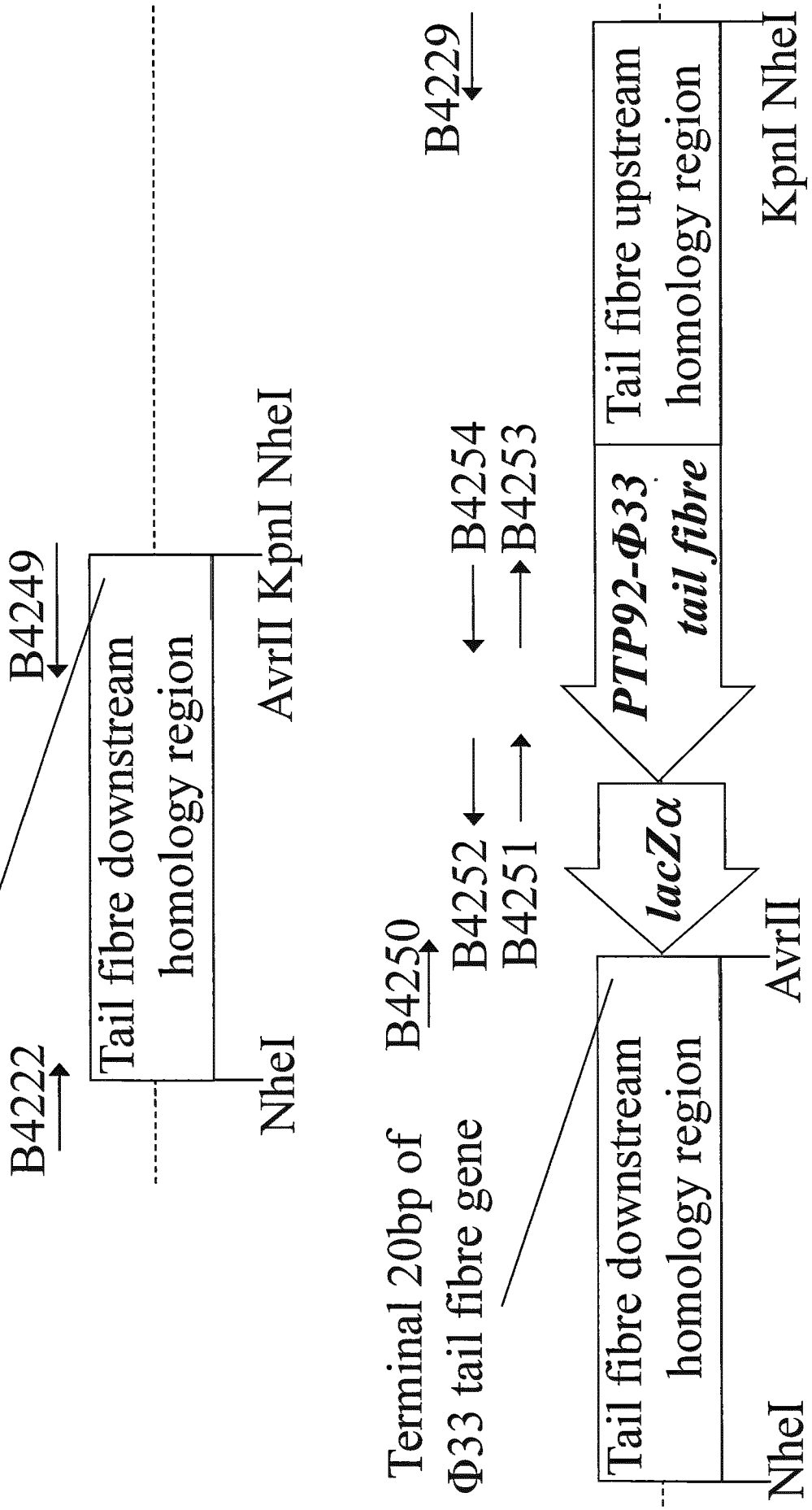


Figure 2 (Part 2 of 4)

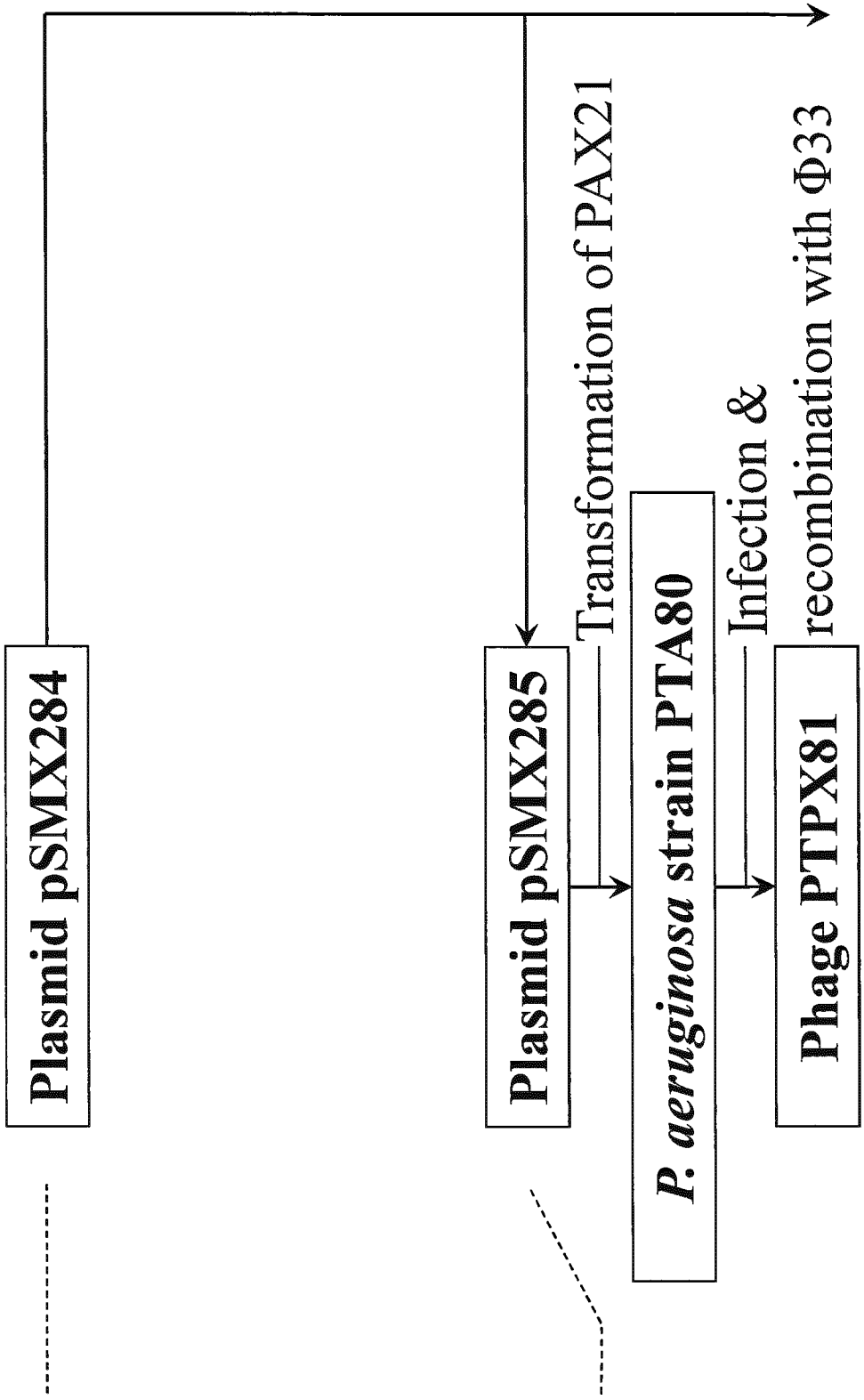


Figure 2 (Part 3 of 4)

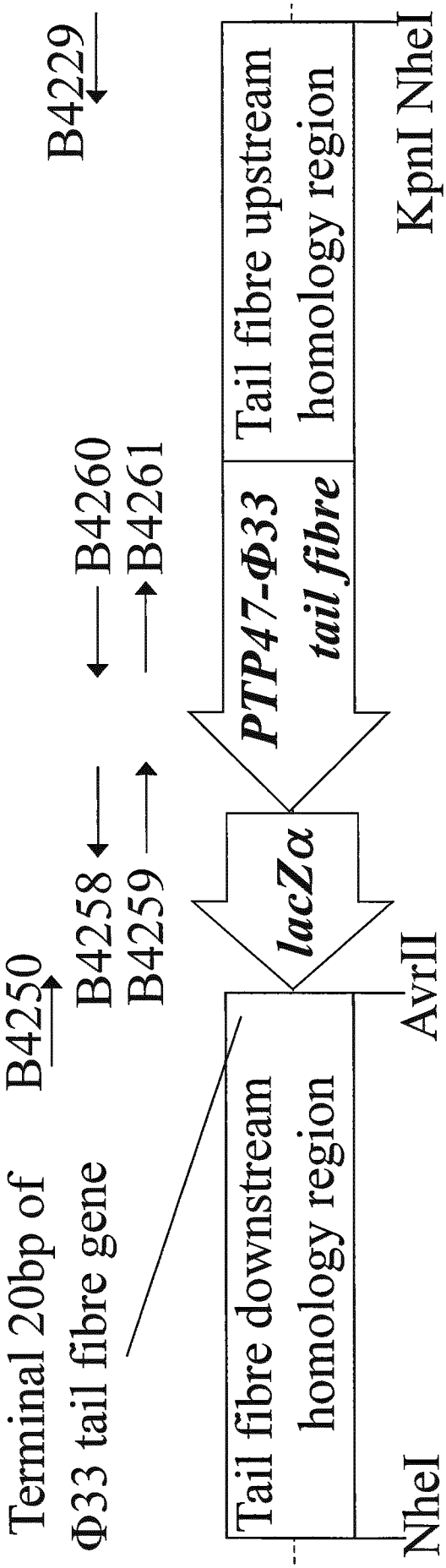
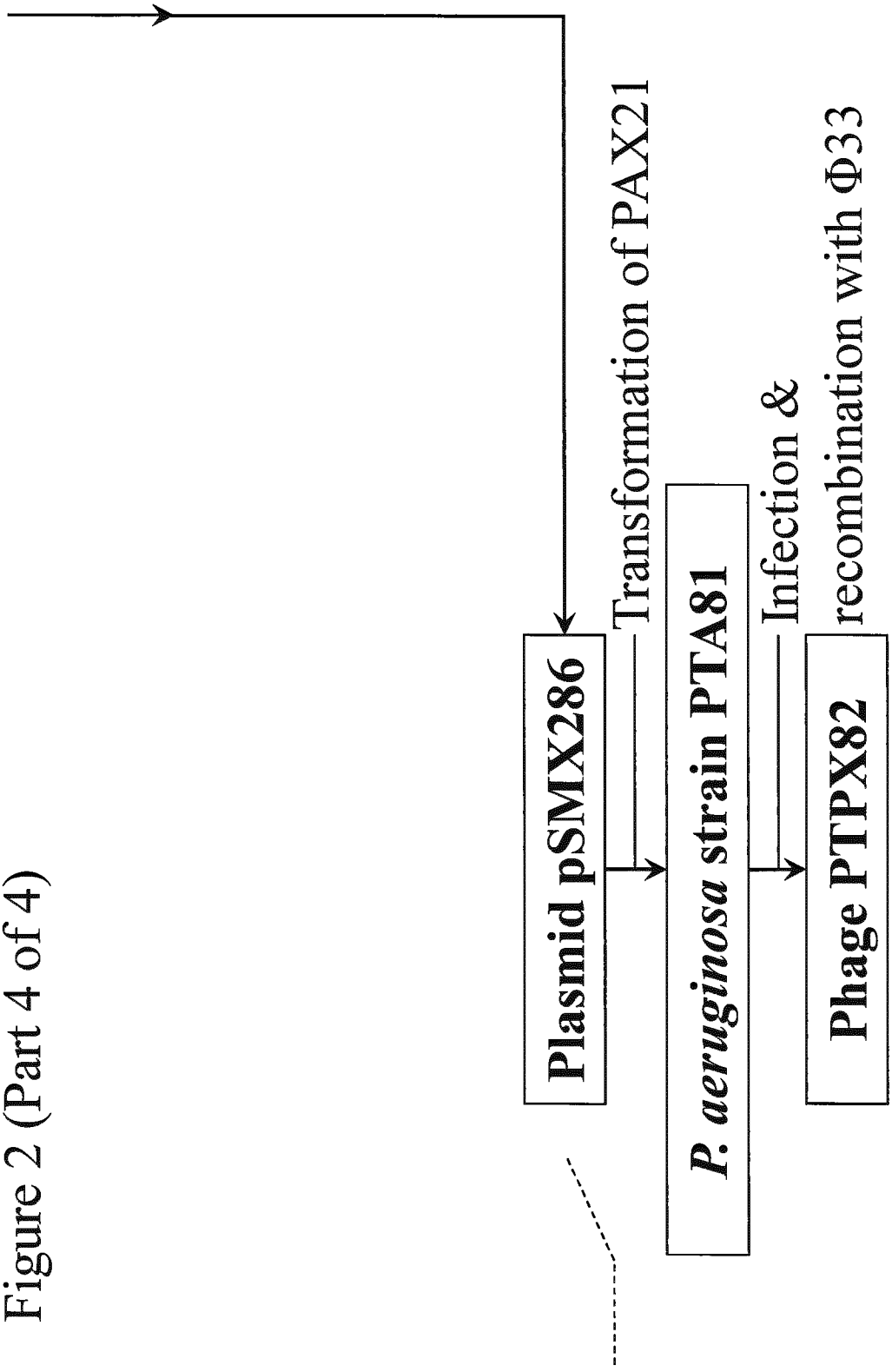


Figure 2 (Part 4 of 4)



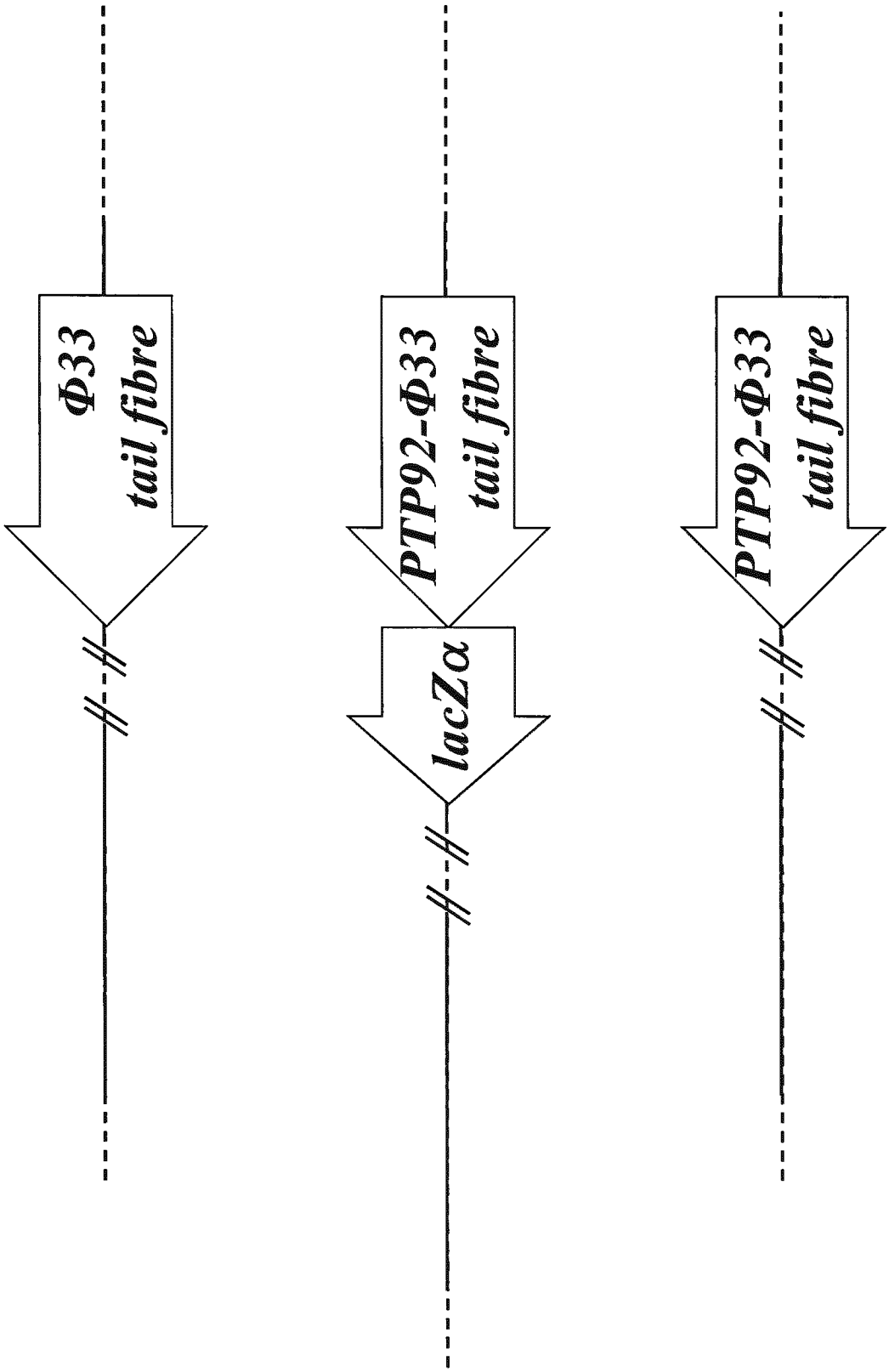
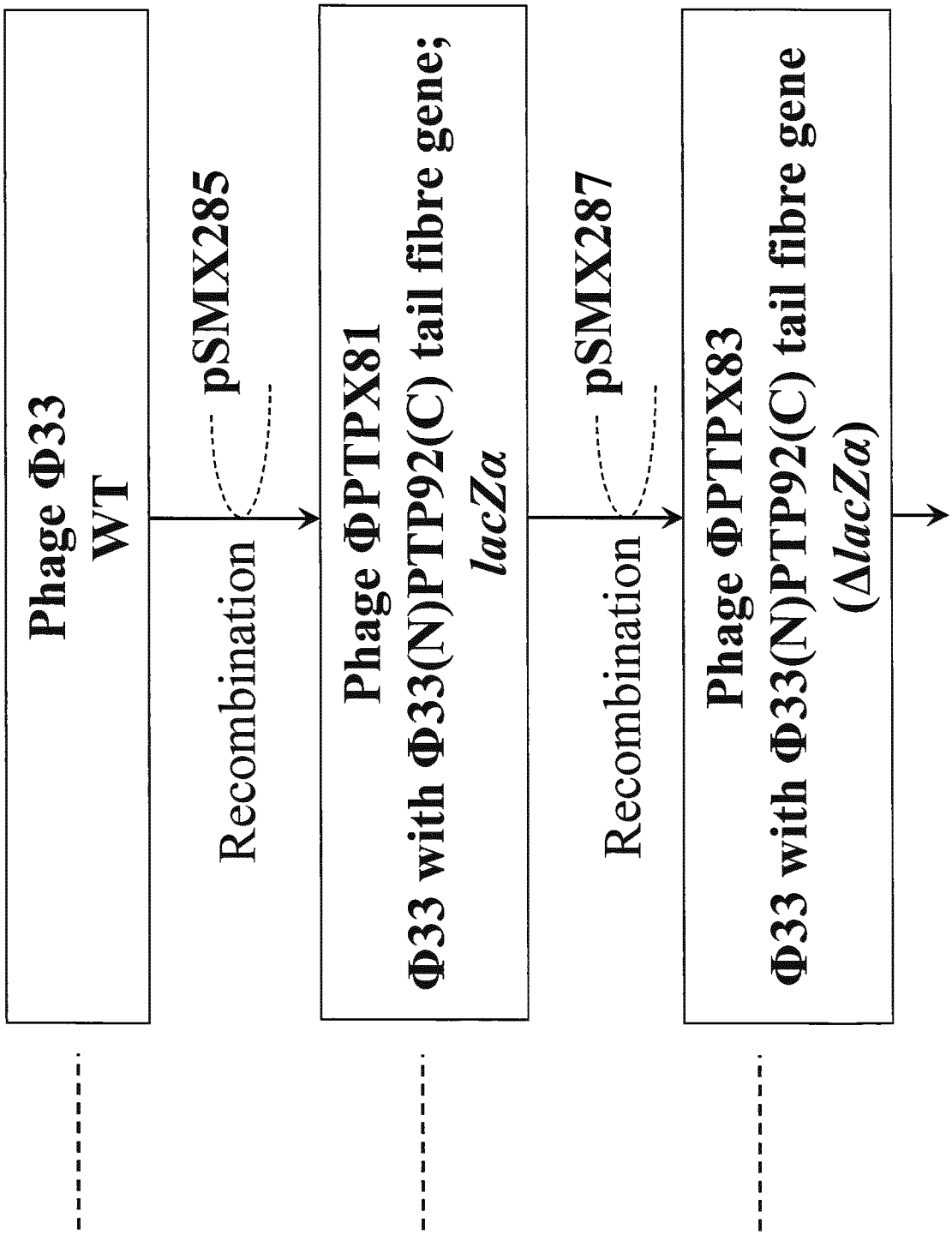


Figure 3 (Part 1 of 4)

Figure 3 (Part 2 of 4)



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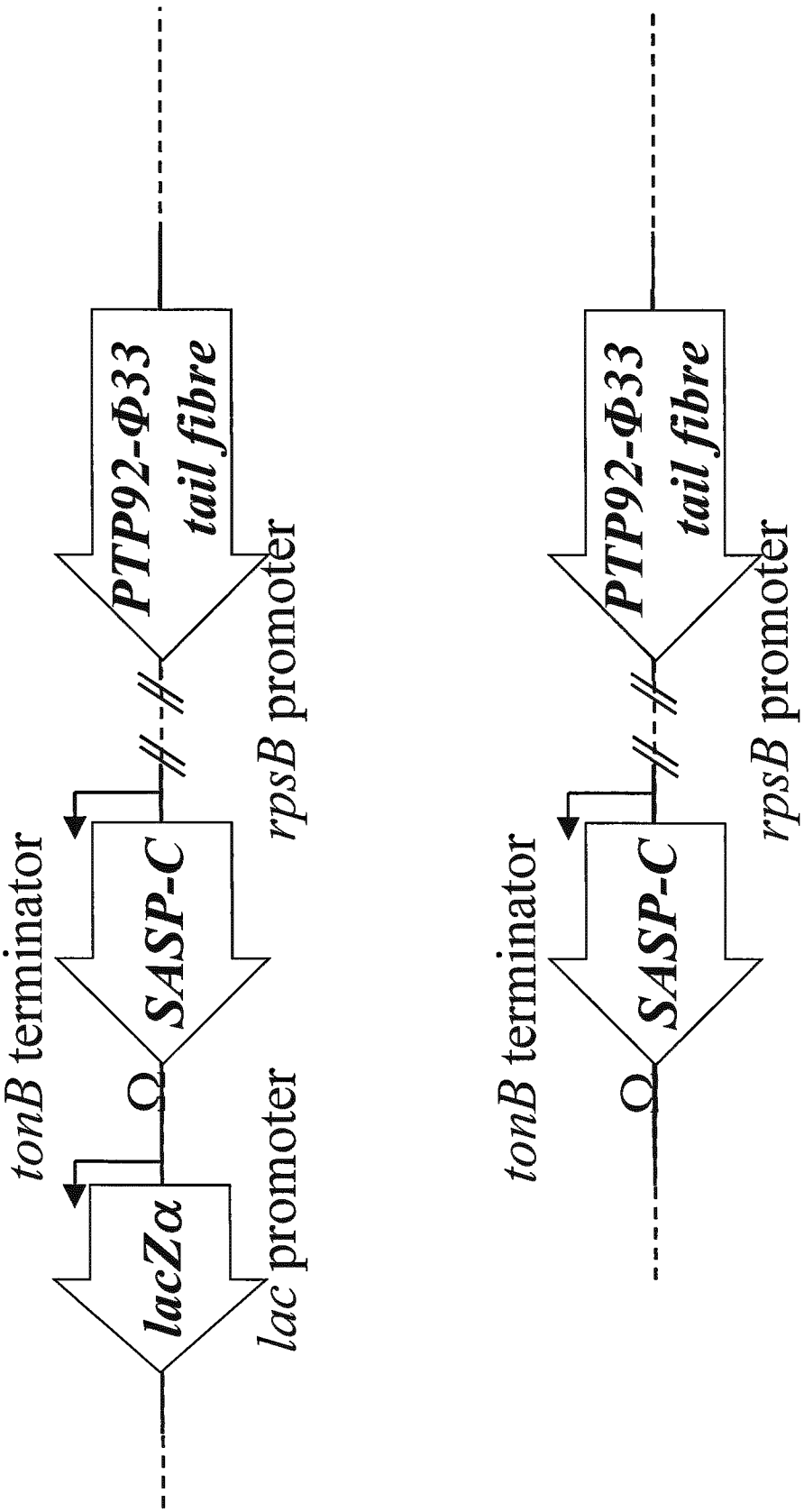

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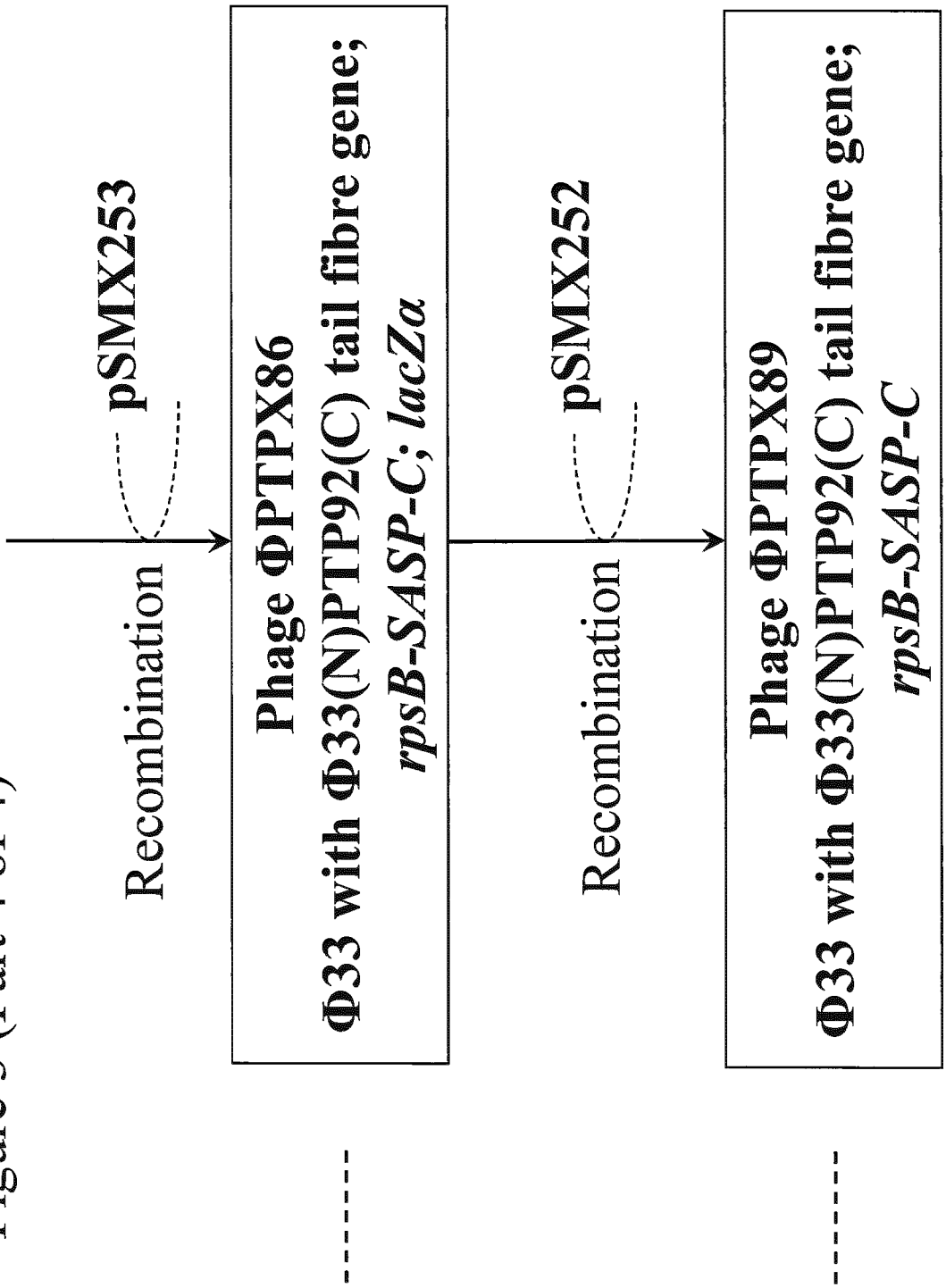


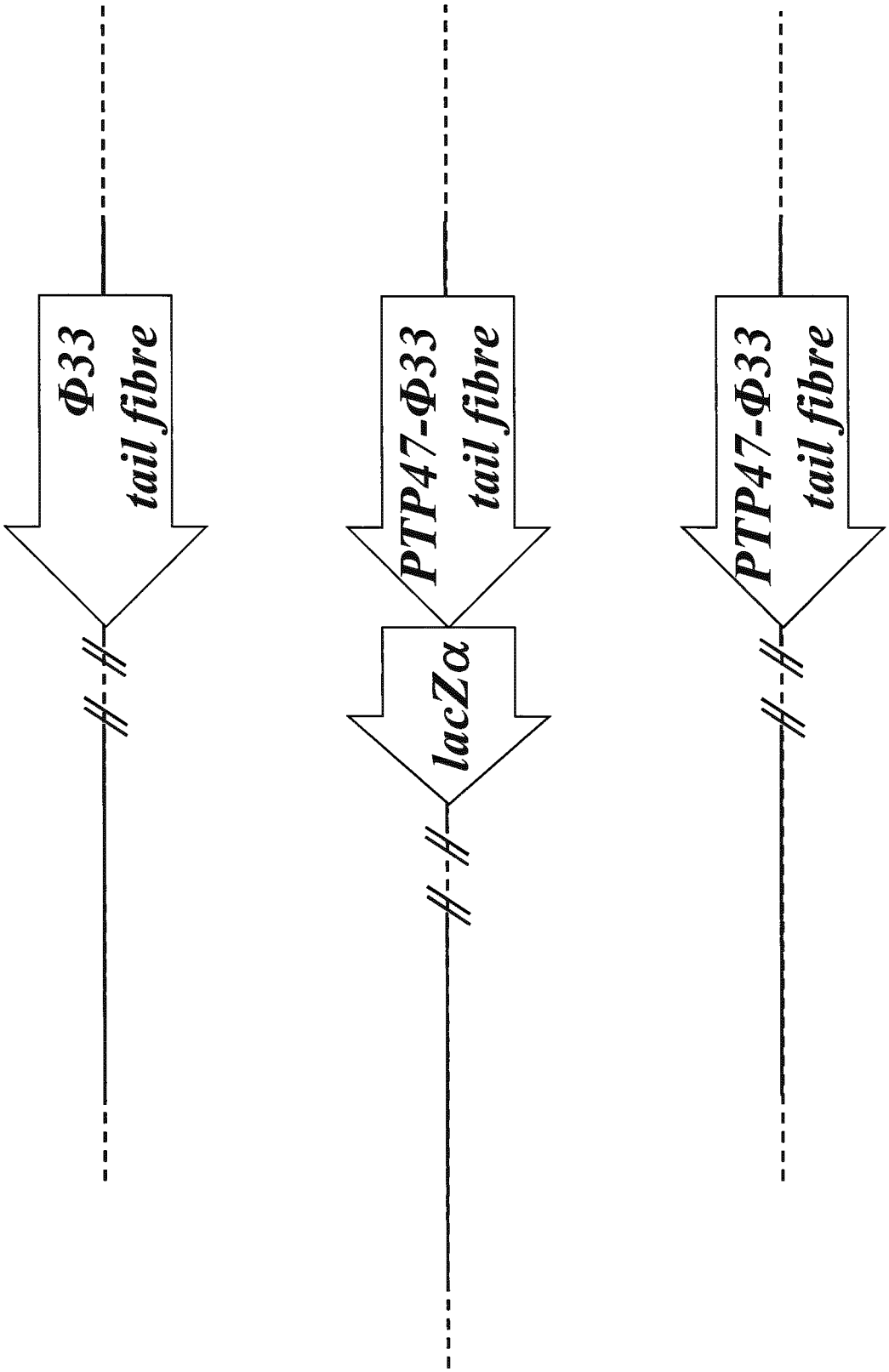
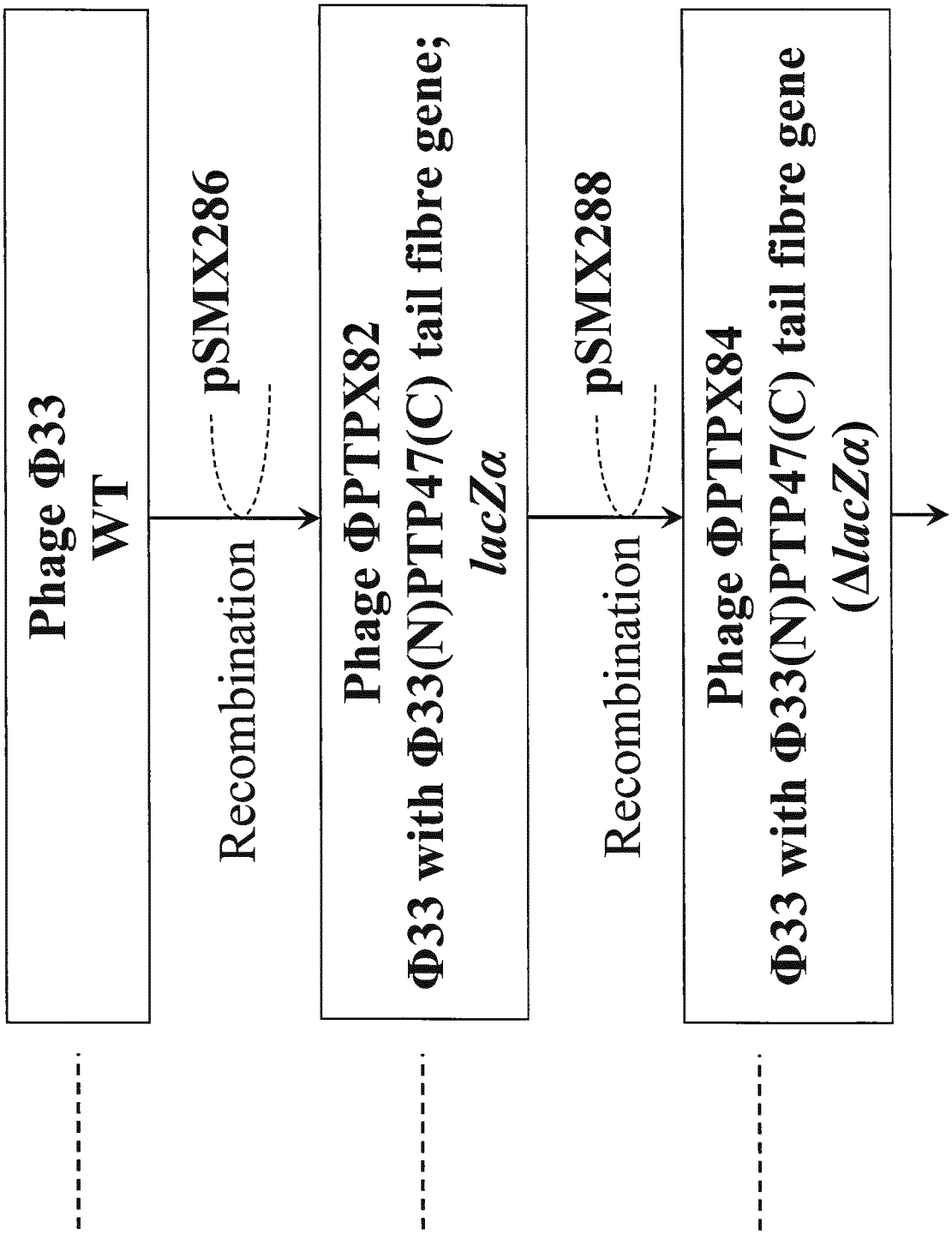


Figure 4 (Part 1 of 4)

Figure 4 (Part 2 of 4)



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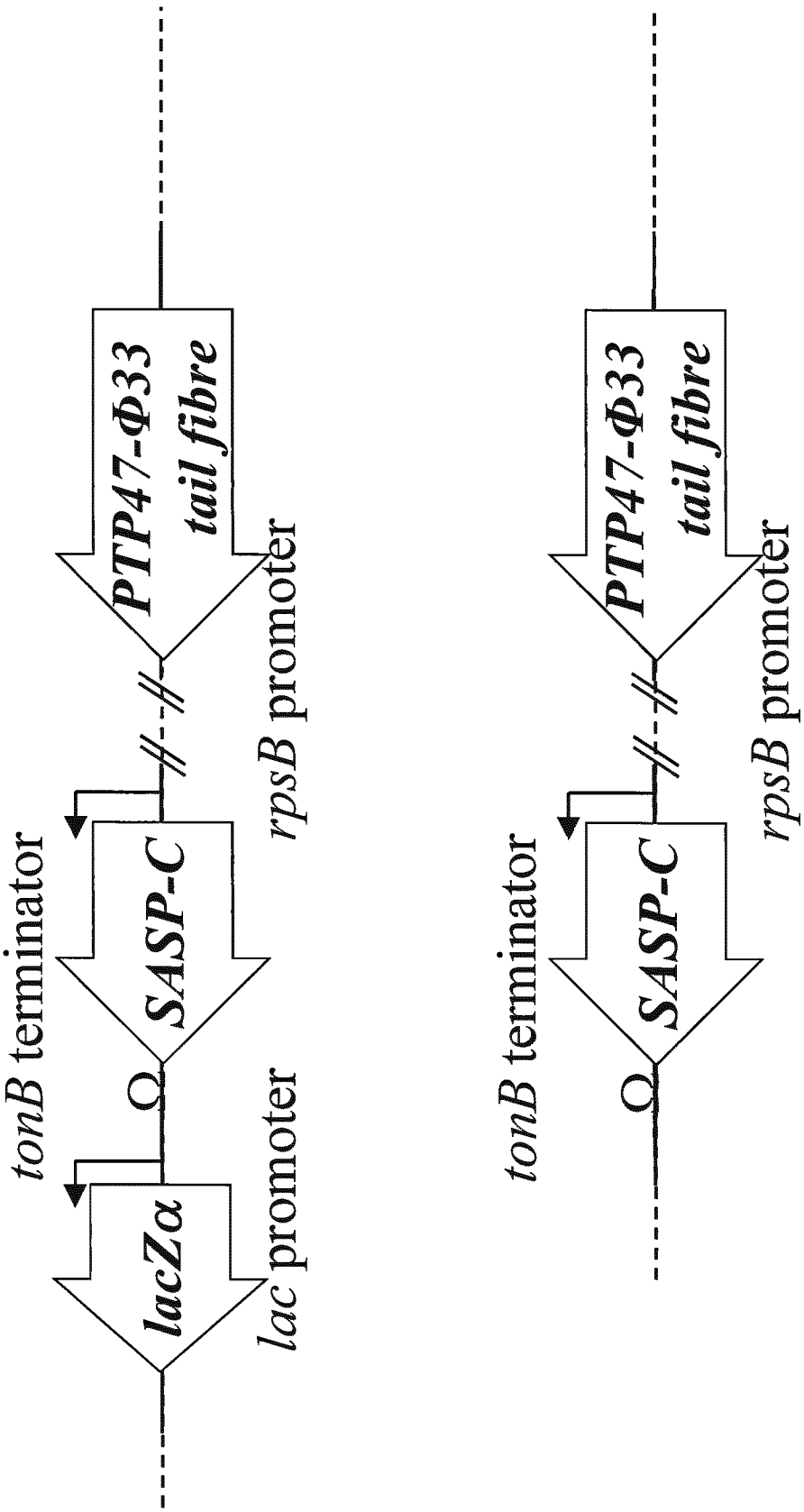

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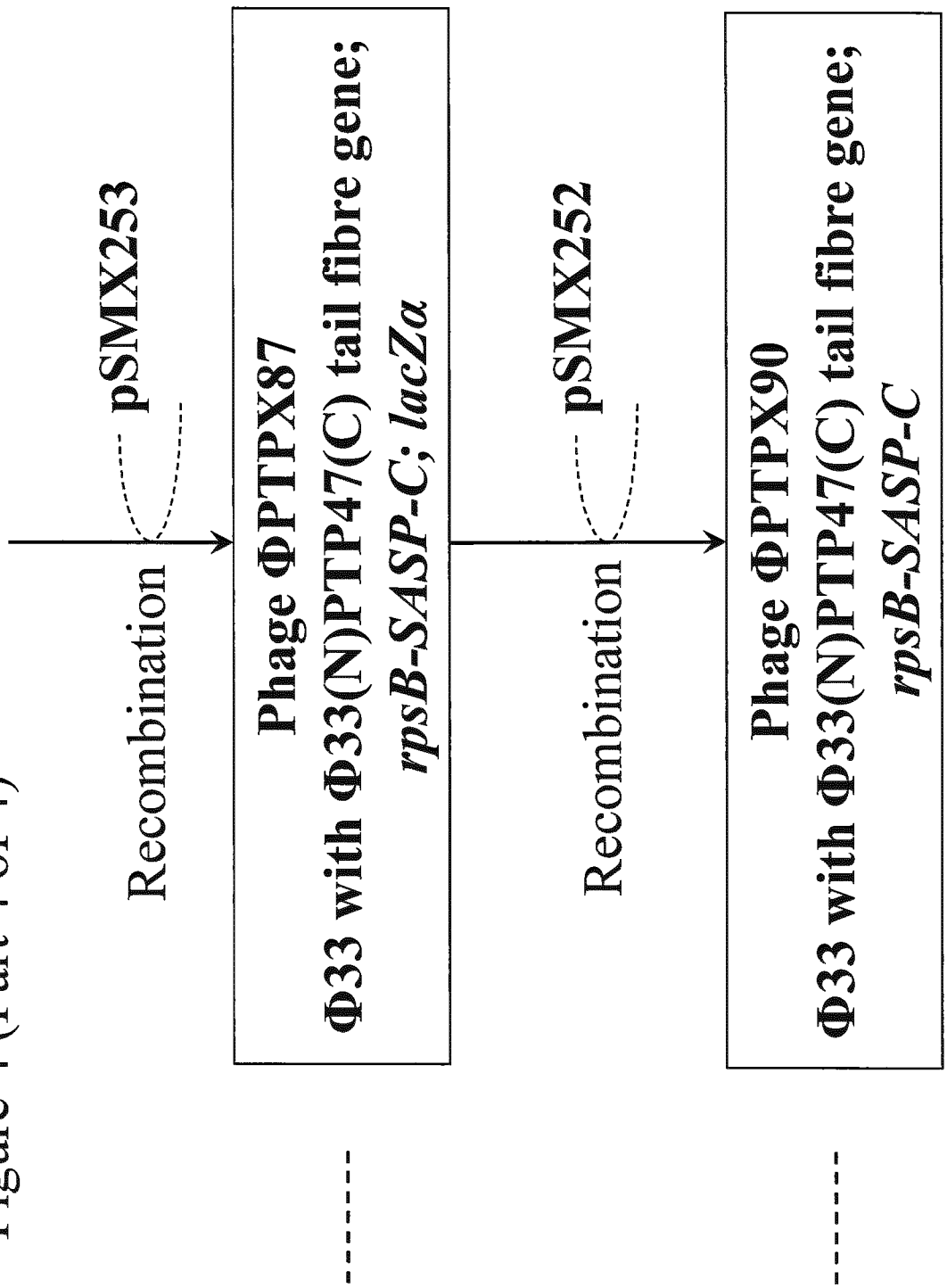

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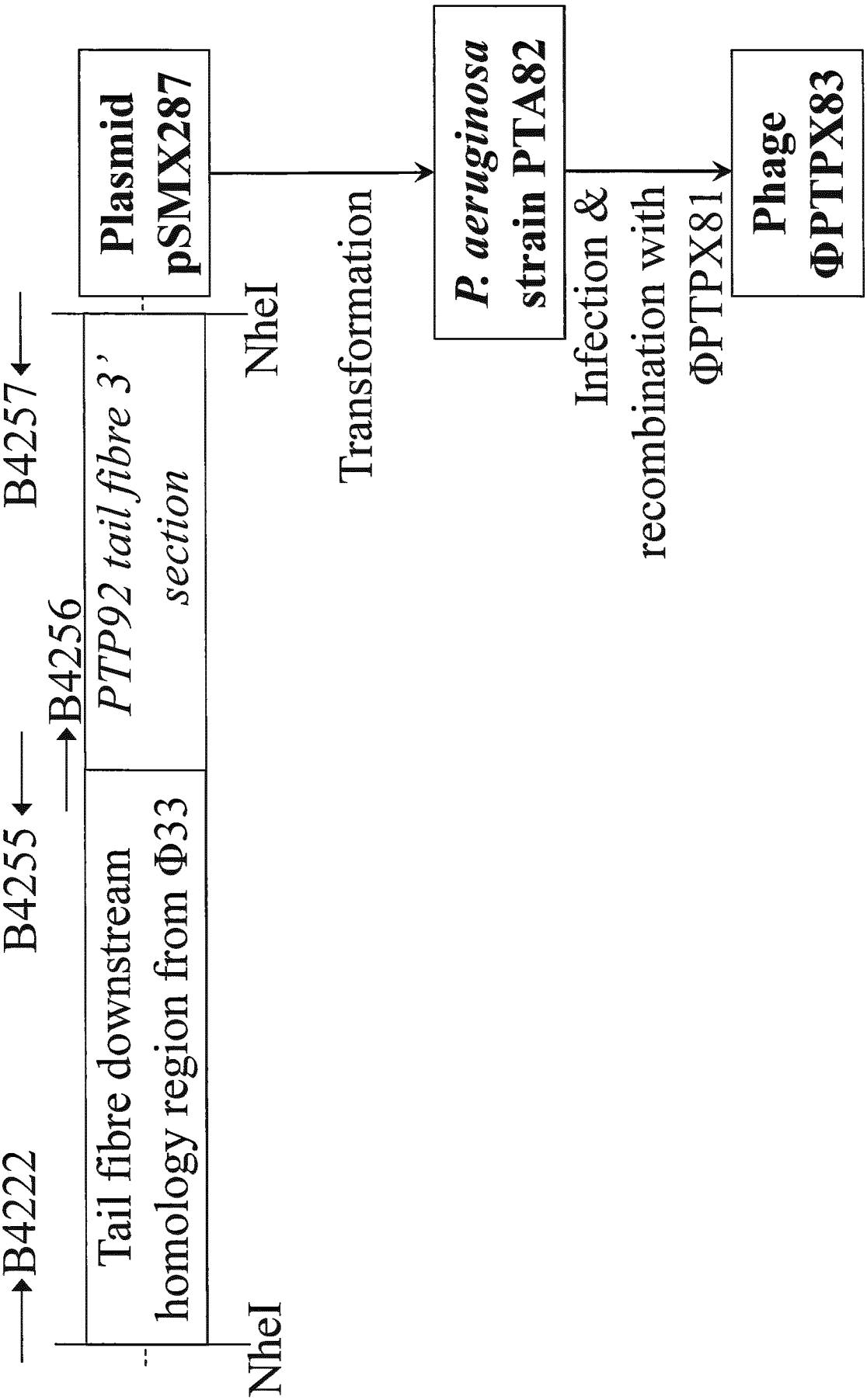
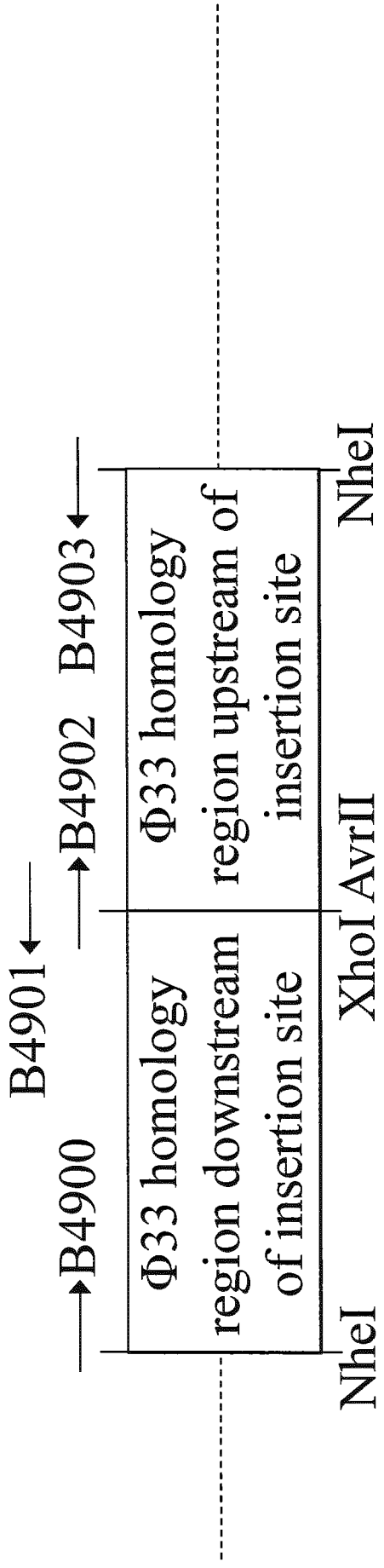
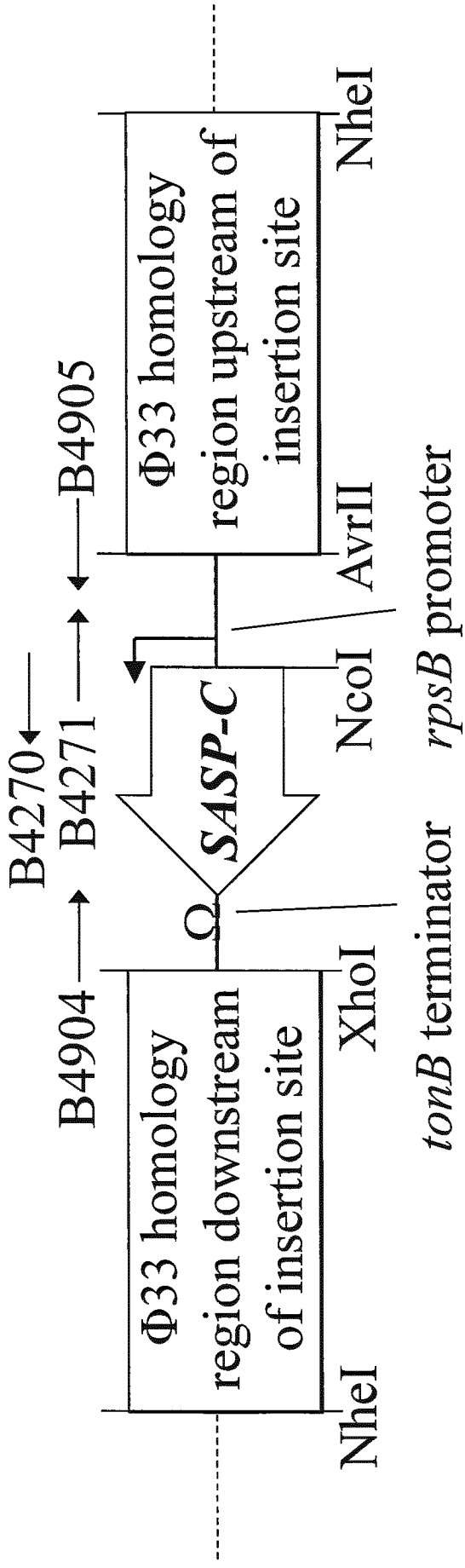


Figure 5 (Part 2 of 2)

Figure 6 (Part 1 of 4)





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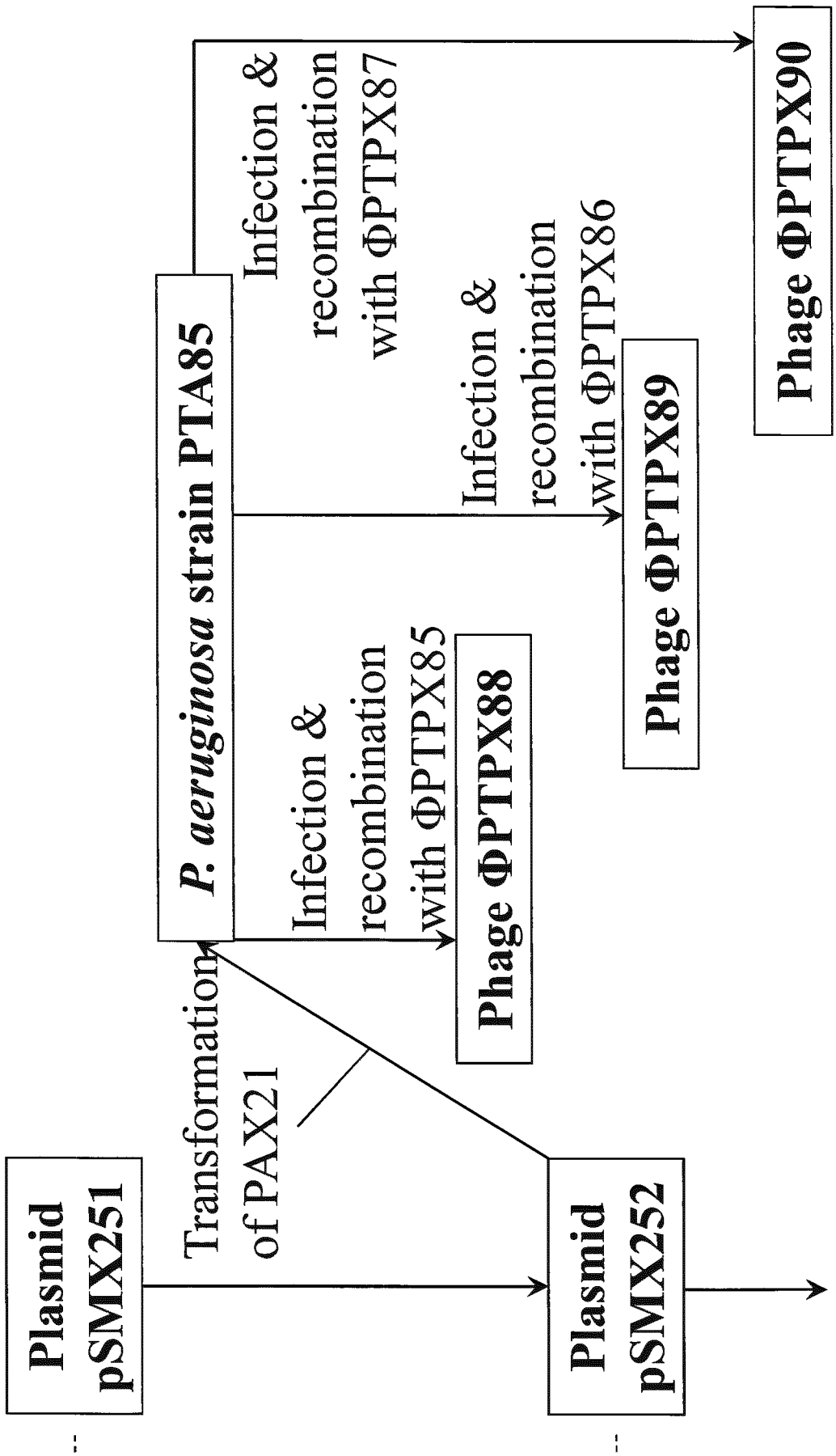
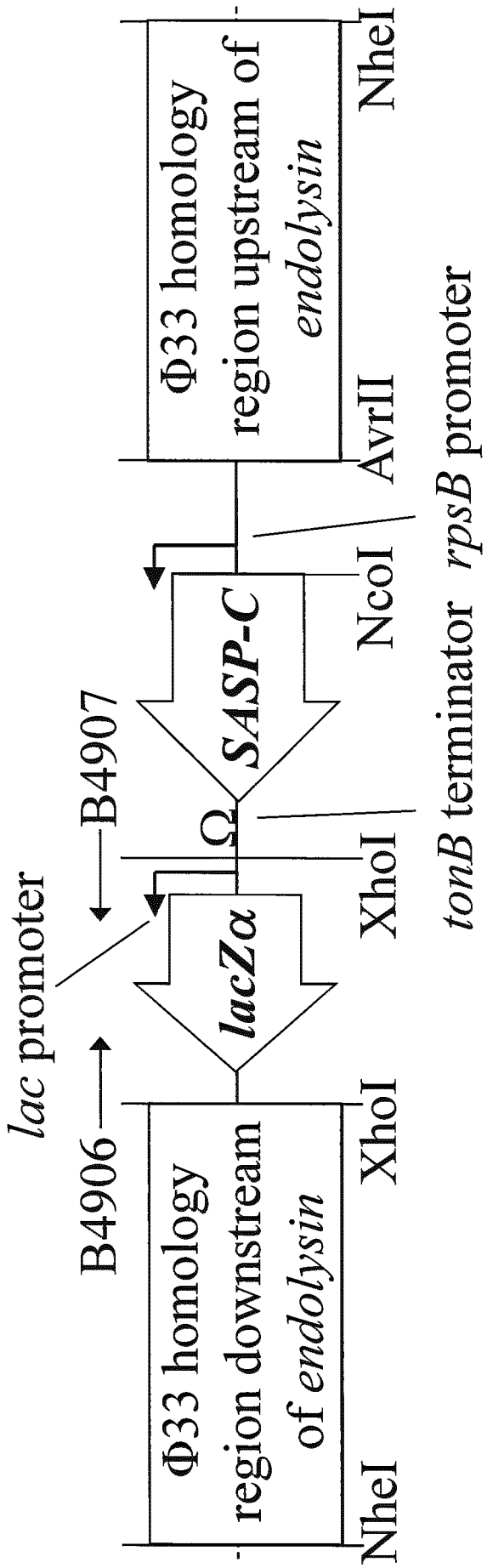


Figure 6 (Part 3 of 4)



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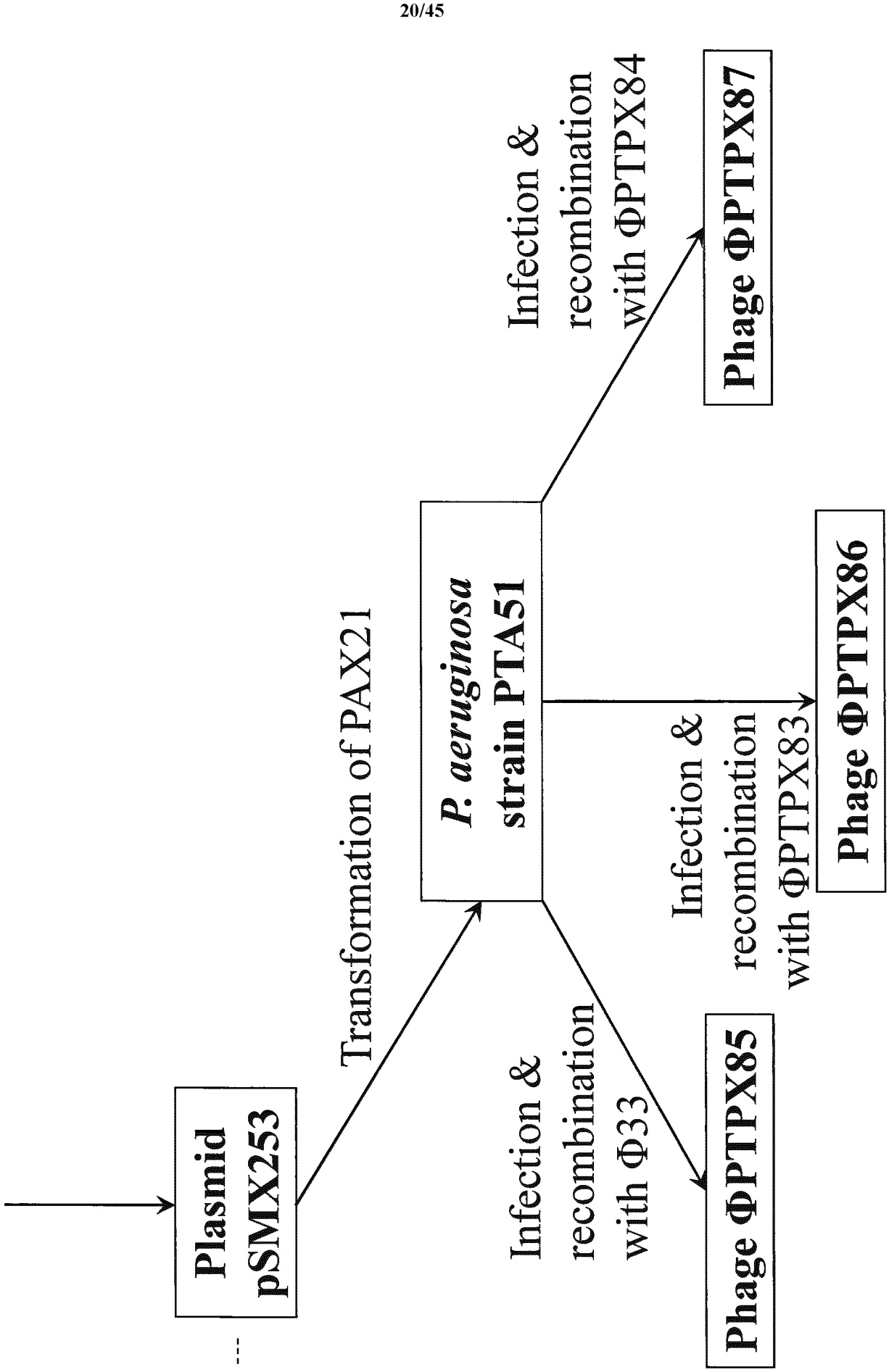

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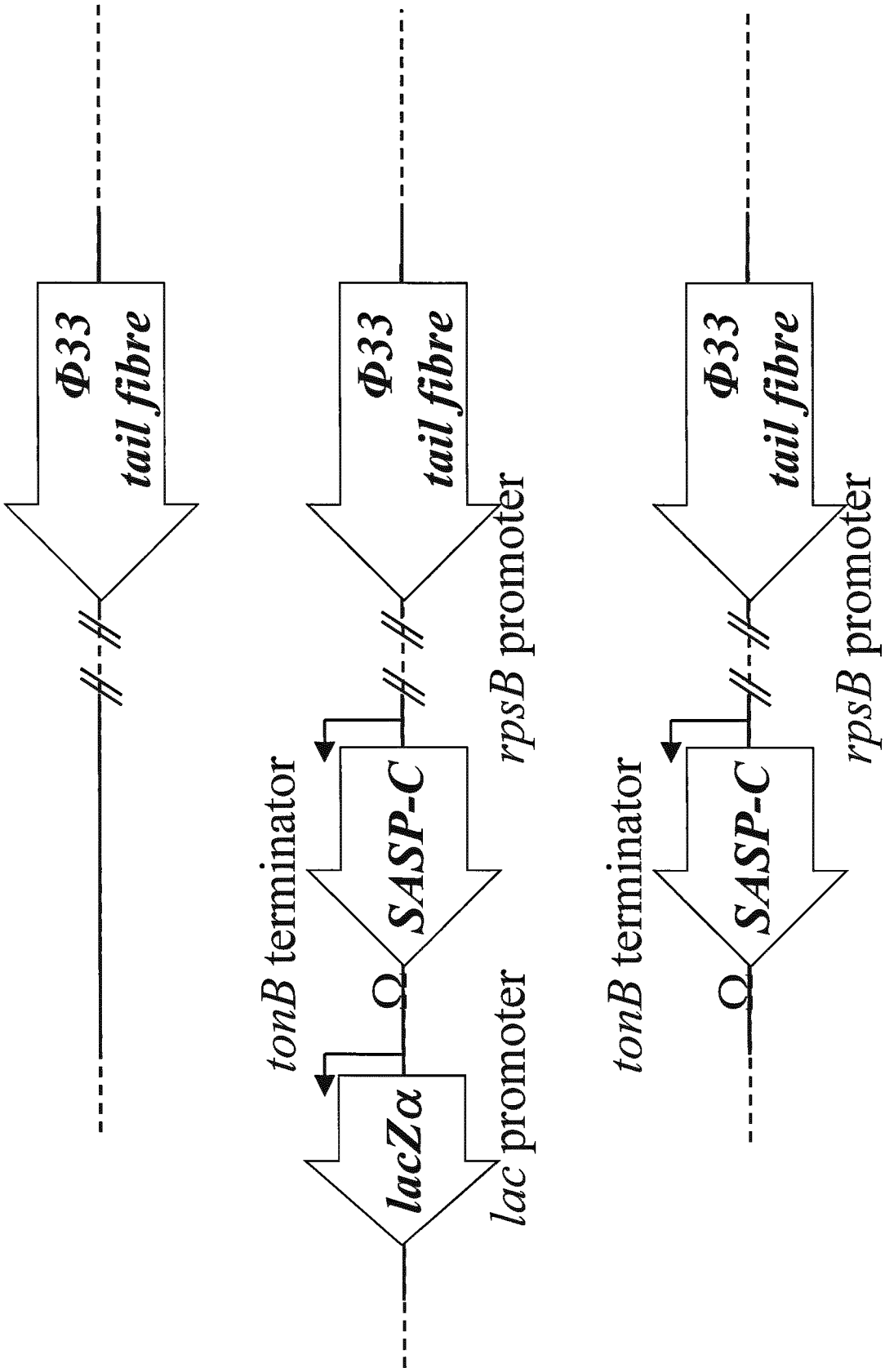


Figure 7 (Part 2 of 2)

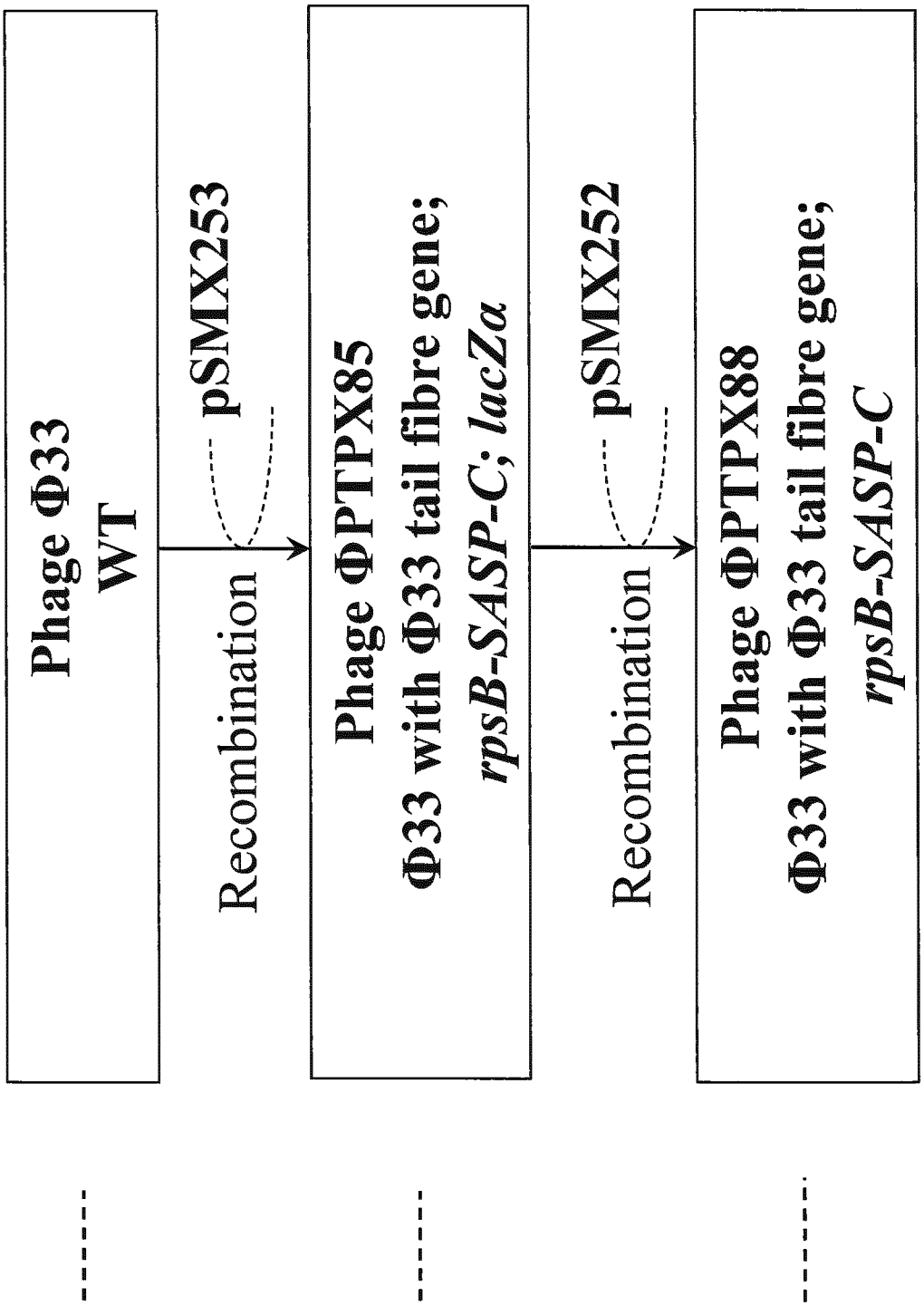
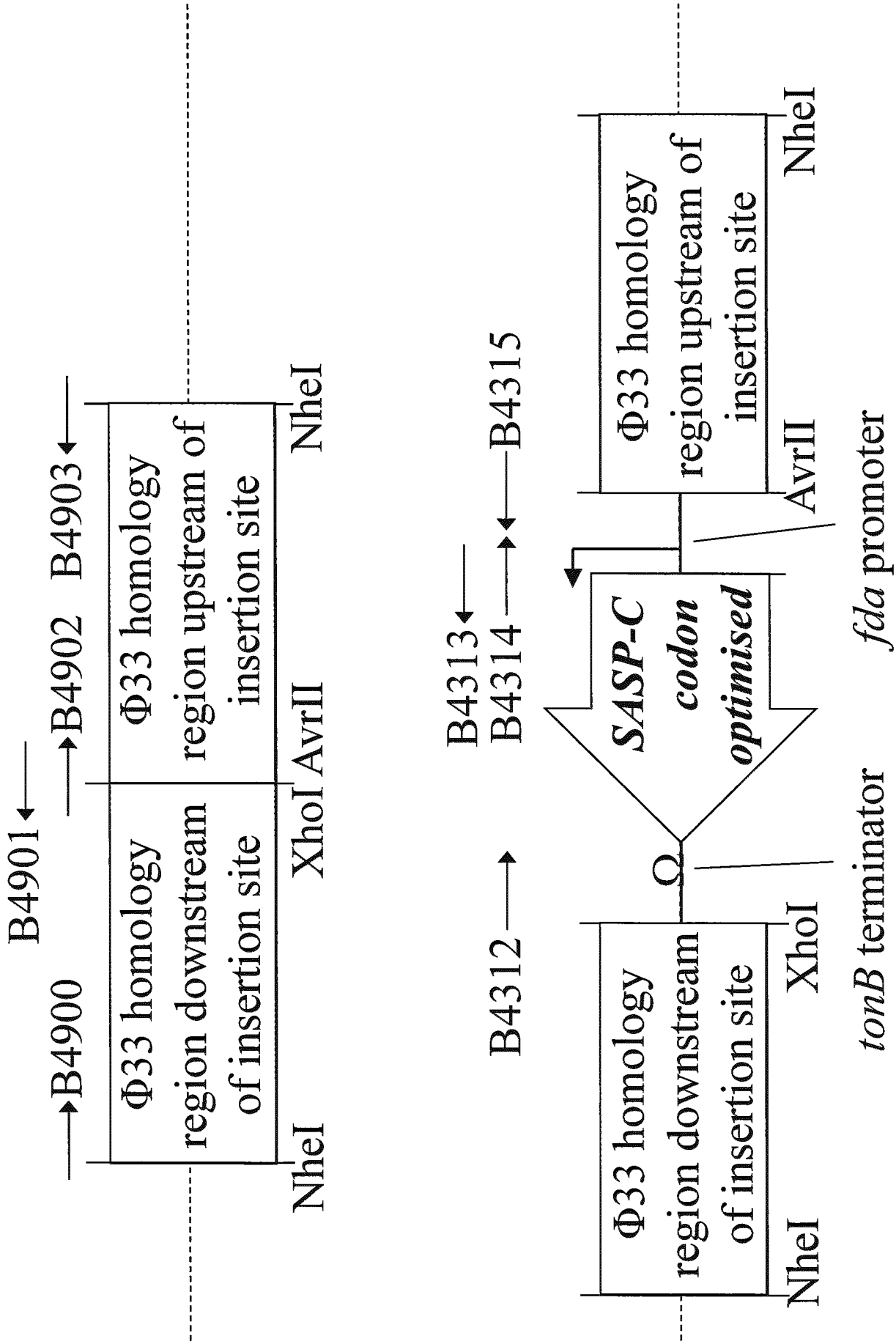


Figure 8 (Part 1 of 4)



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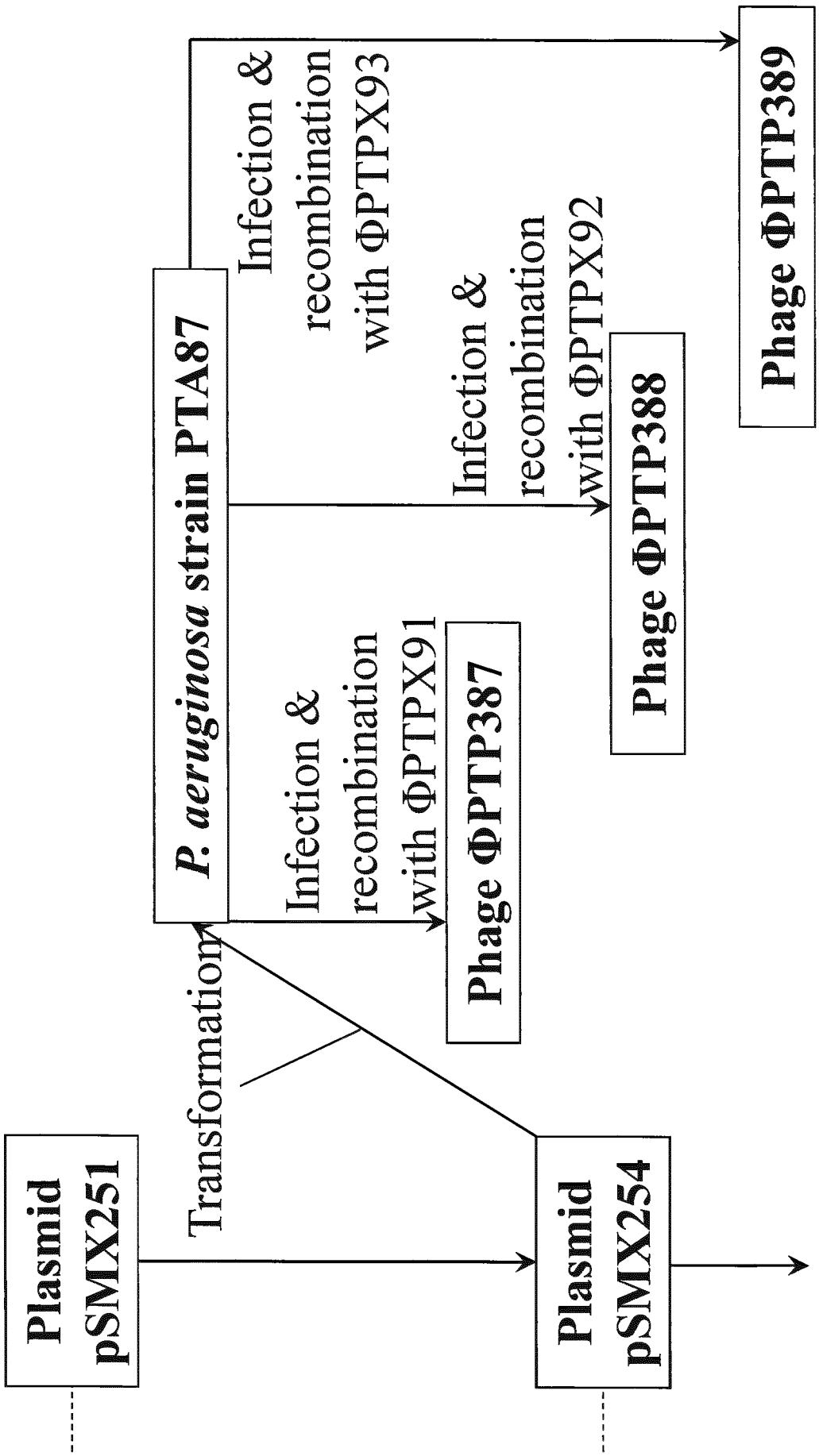
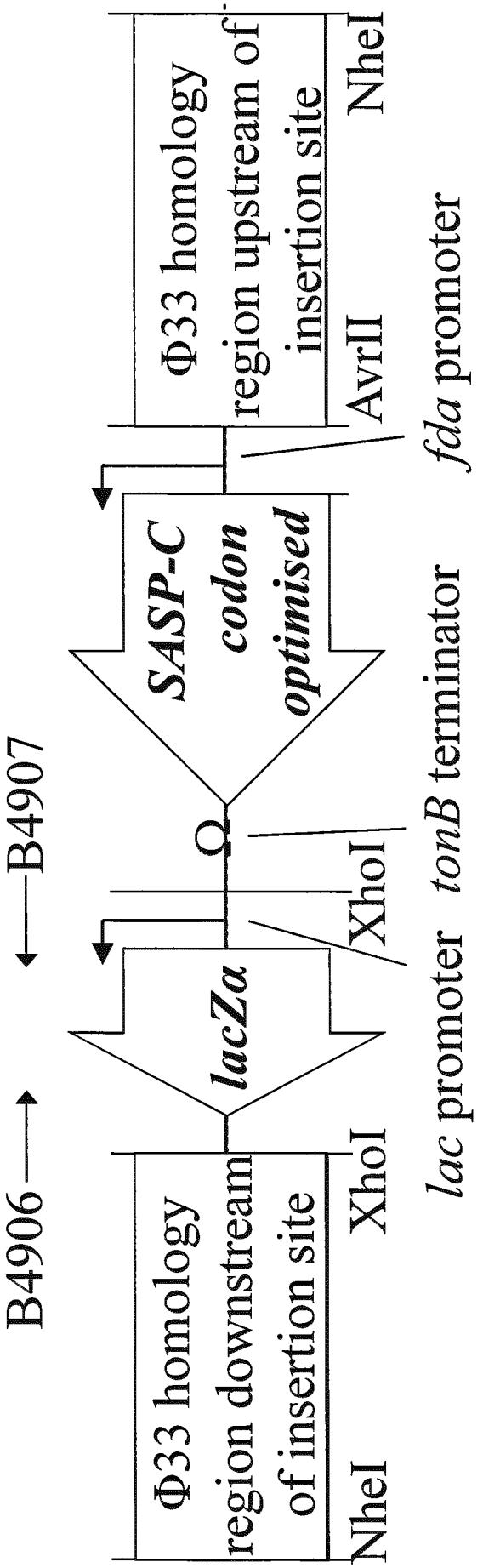


Figure 8 (Part 3 of 4)



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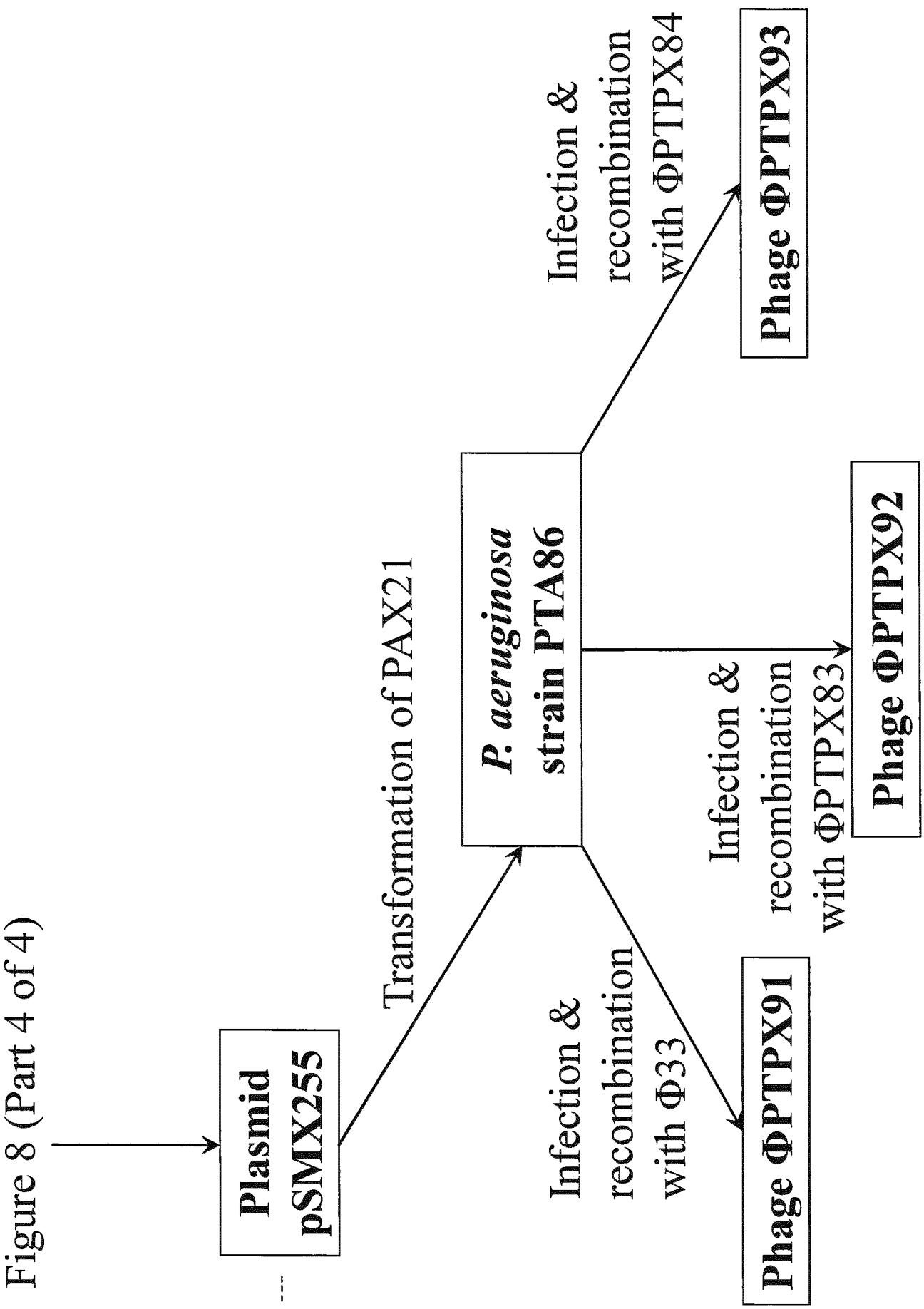


Figure 9

>codon_optimised_SASP-C
ATGGCCAACTACCAGAACGCGAGCAACCGCAACAGCAGCAAC
AAGCTGGTCGCGCCGGGCGCCCAGGCCGCCATCGACCAGATG
AAGTTCGAGATCGCGAGCGAGTTCGGCGCGTGAACCTCGGCCCG
GACGCCACCGCCCCGTGCCAACGGCTCGGTCGGCGCGAAATC
ACCAAGCGCCTGGTGCAGCTGGCGCGGAACAGAACCTGGGCGGC
AAGTACTGA

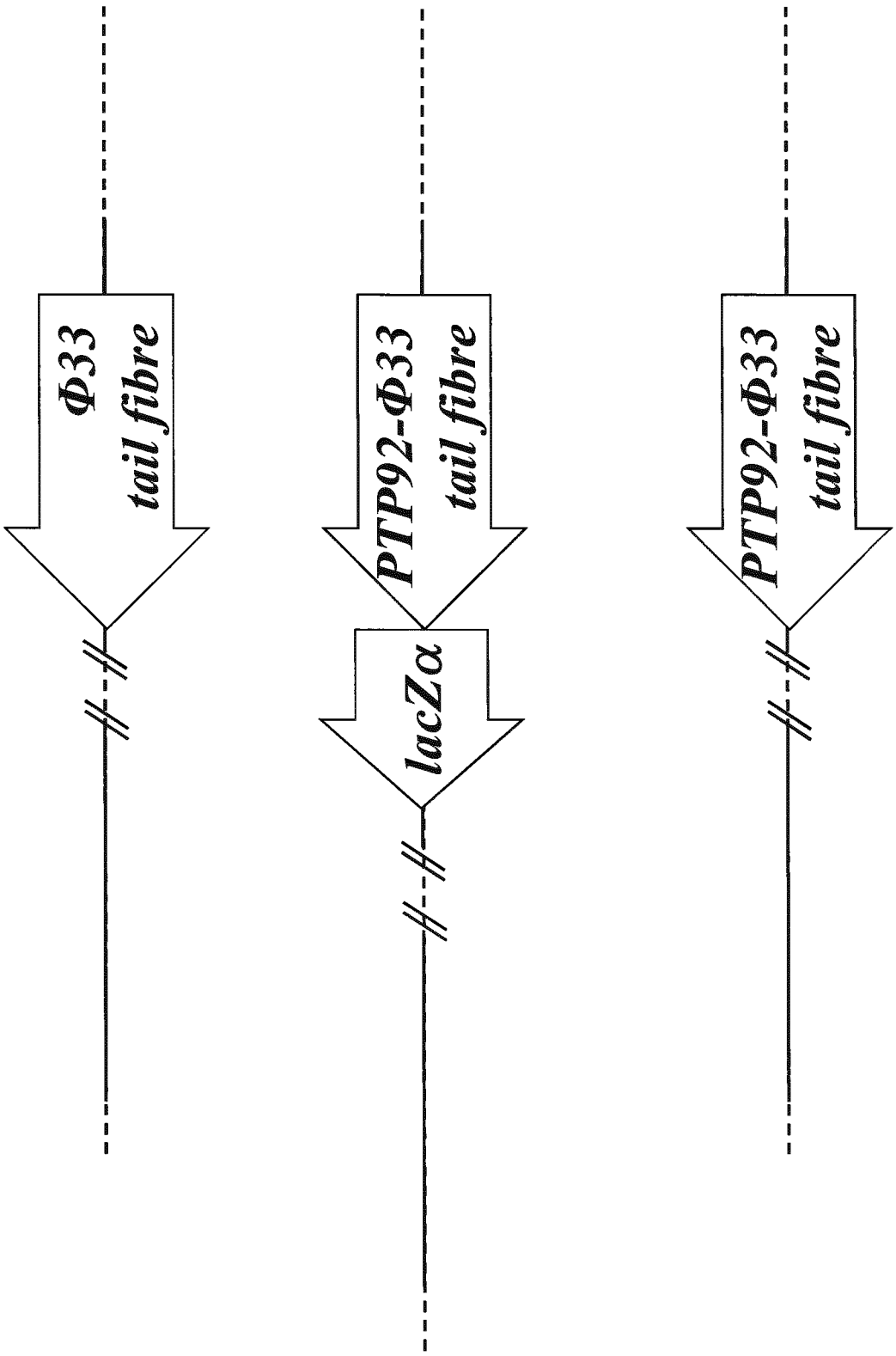


Figure 10 (Part 1 of 4)

Figure 10 (Part 2 of 4)

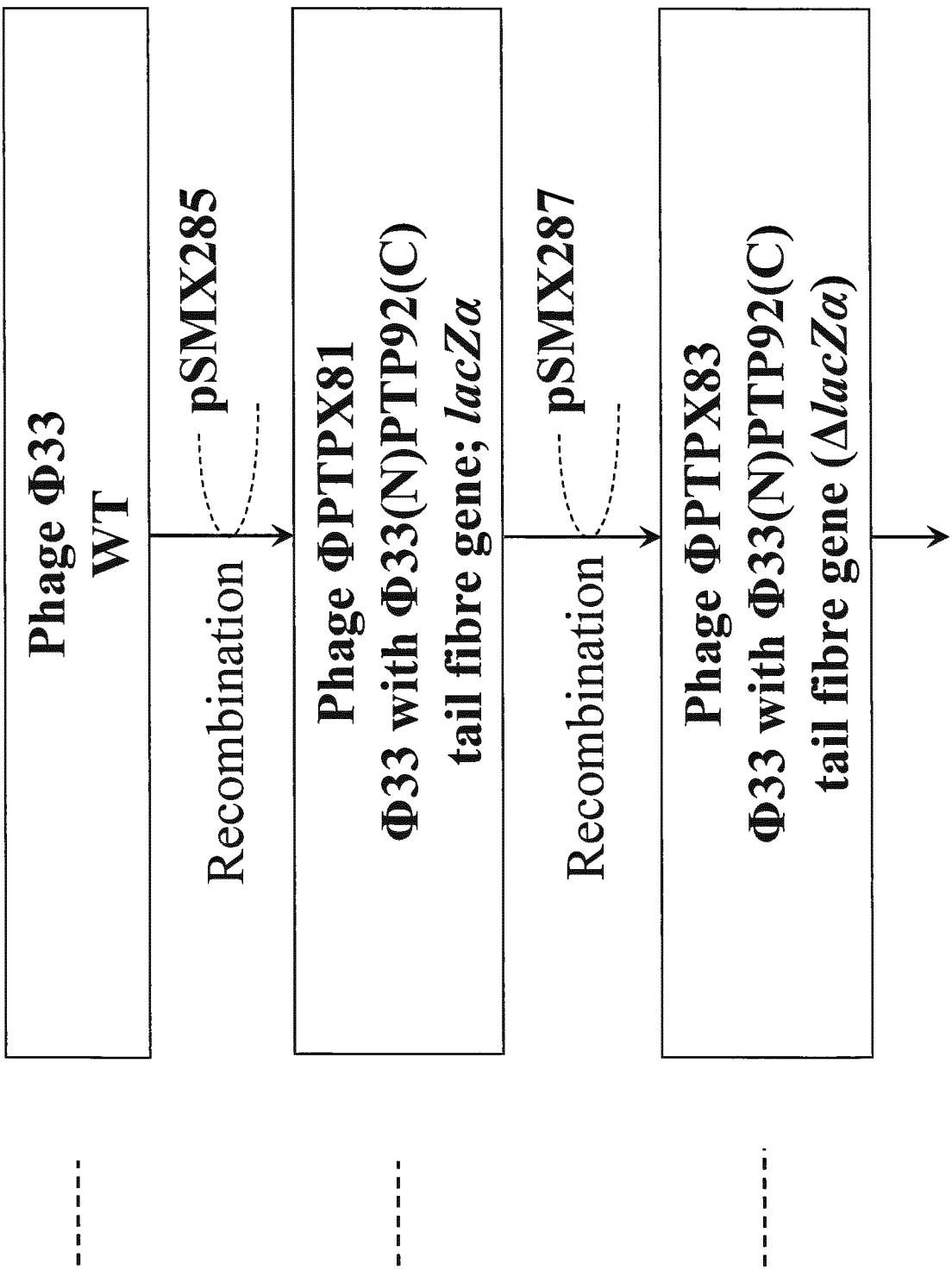


Figure 10 (Part 3 of 4)

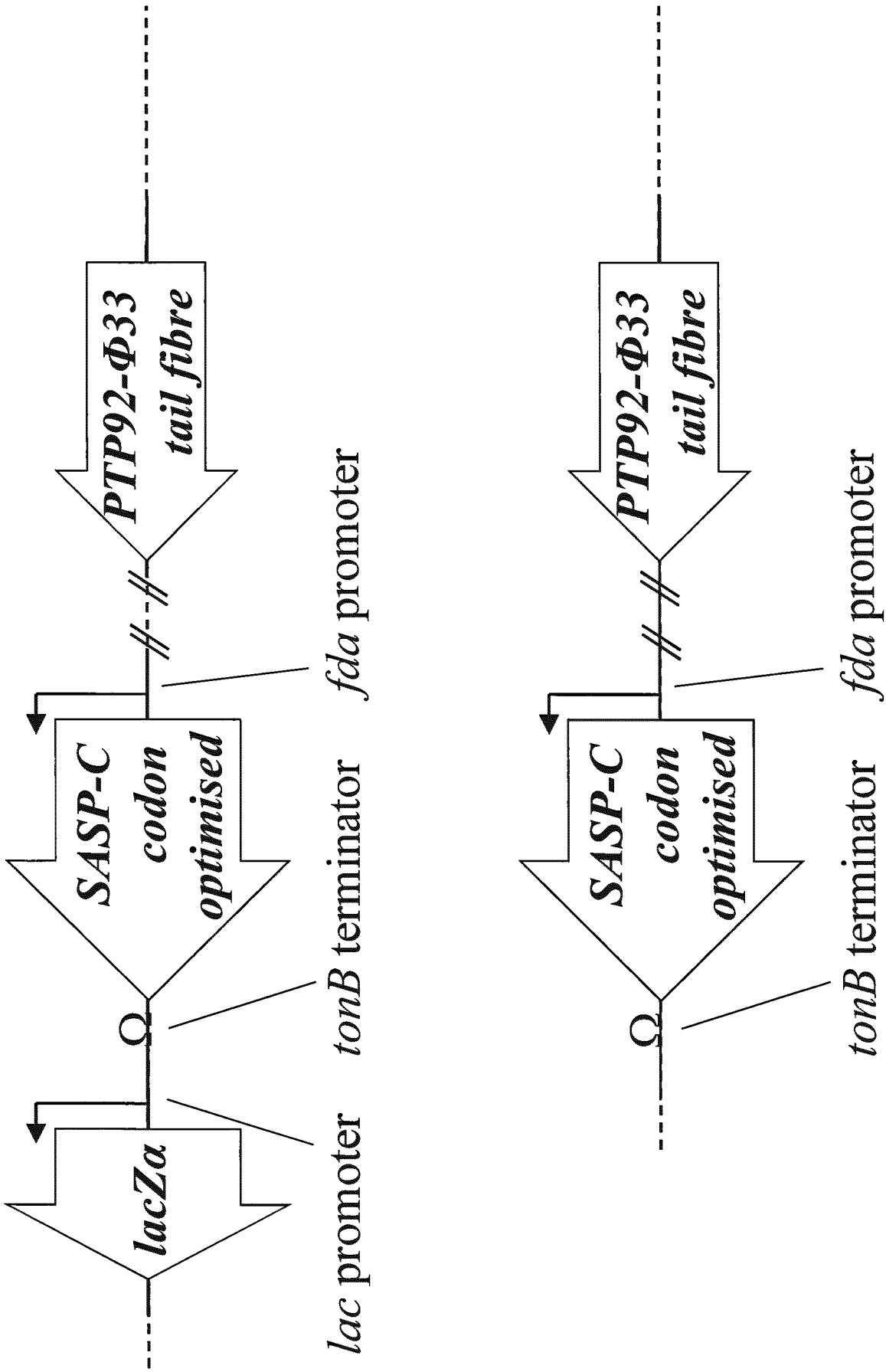
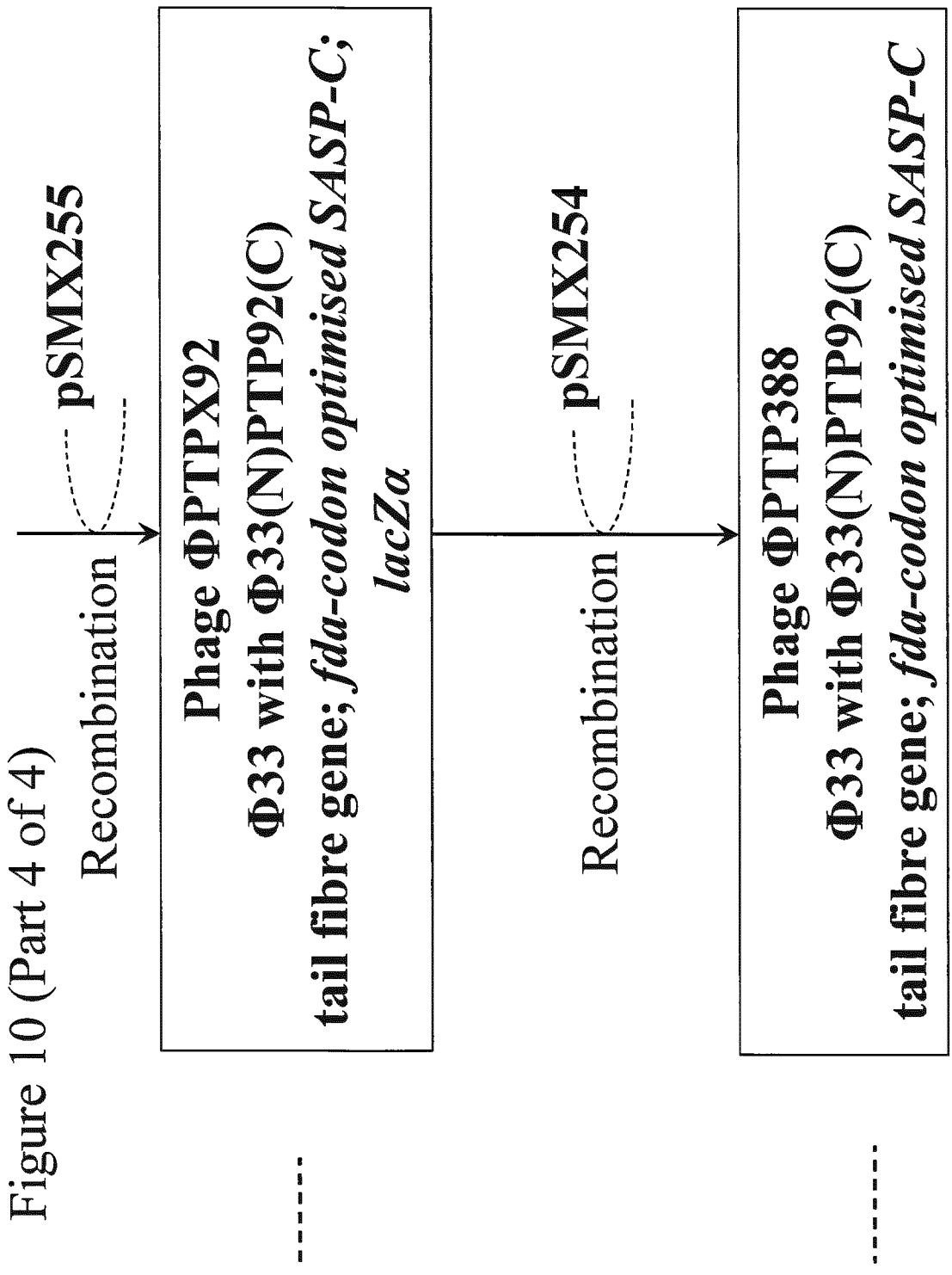


Figure 10 (Part 4 of 4)



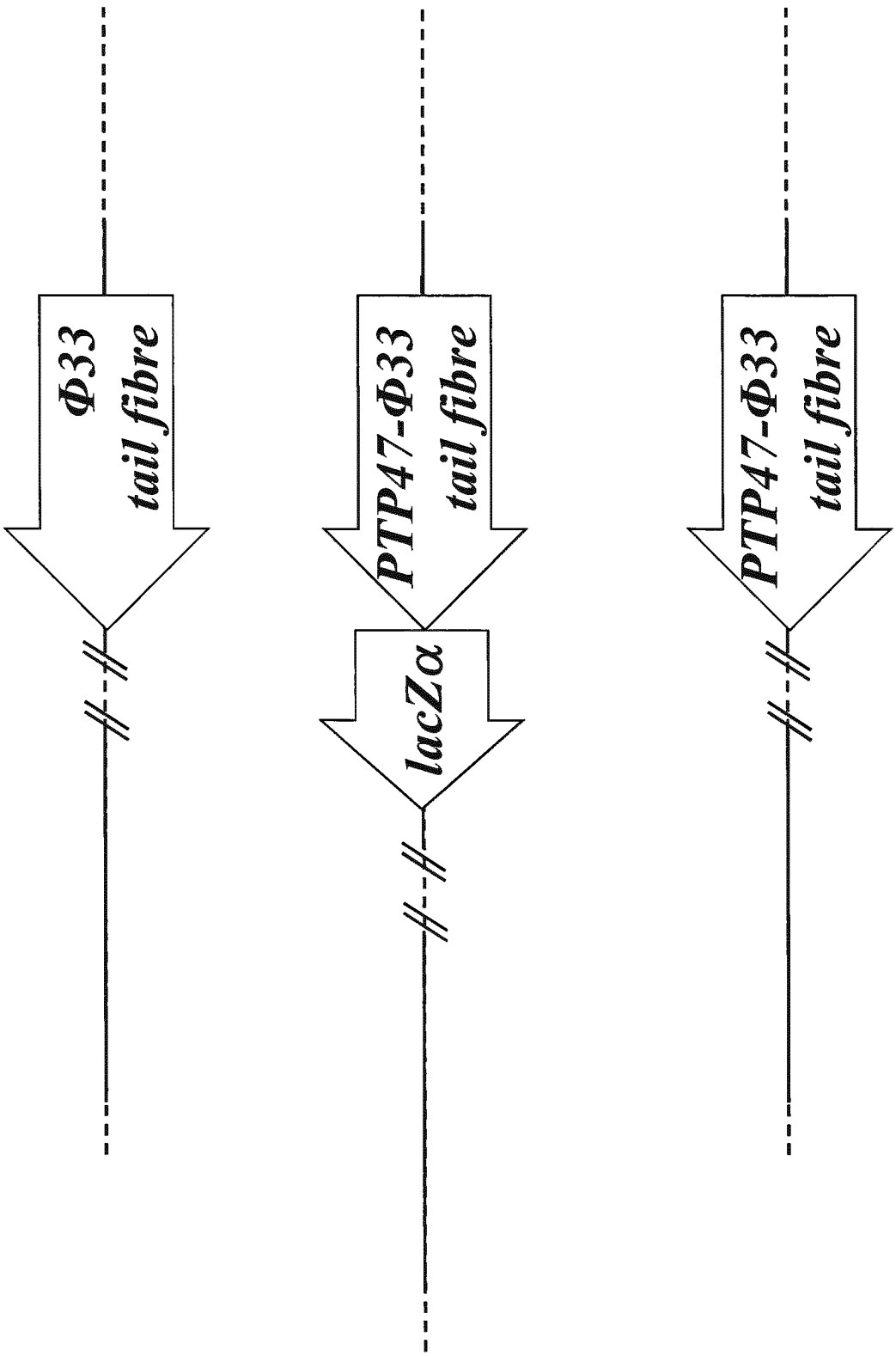


Figure 11 (Part 1 of 4)

Figure 11 (Part 2 of 4)

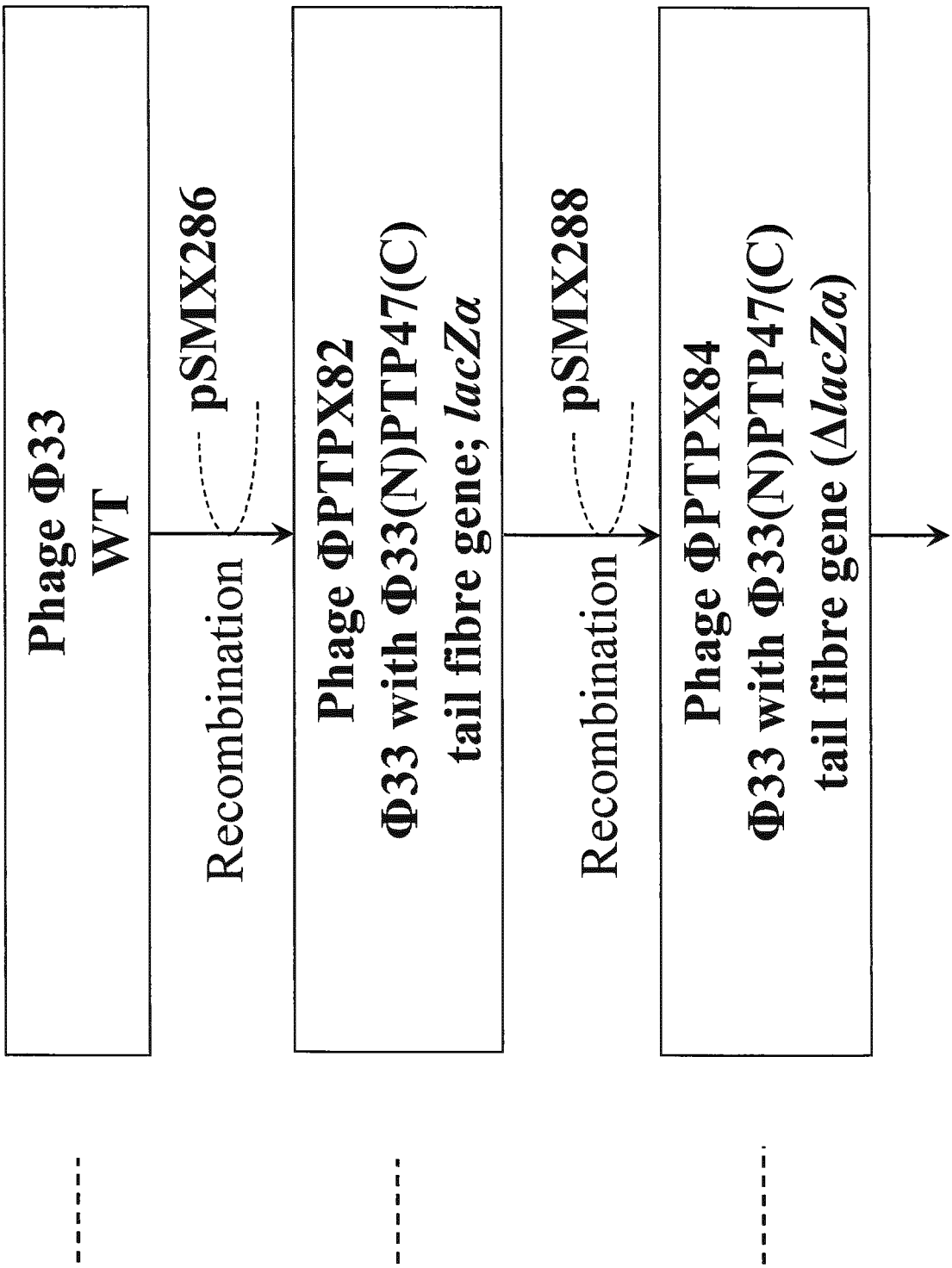


Figure 11 (Part 3 of 4)

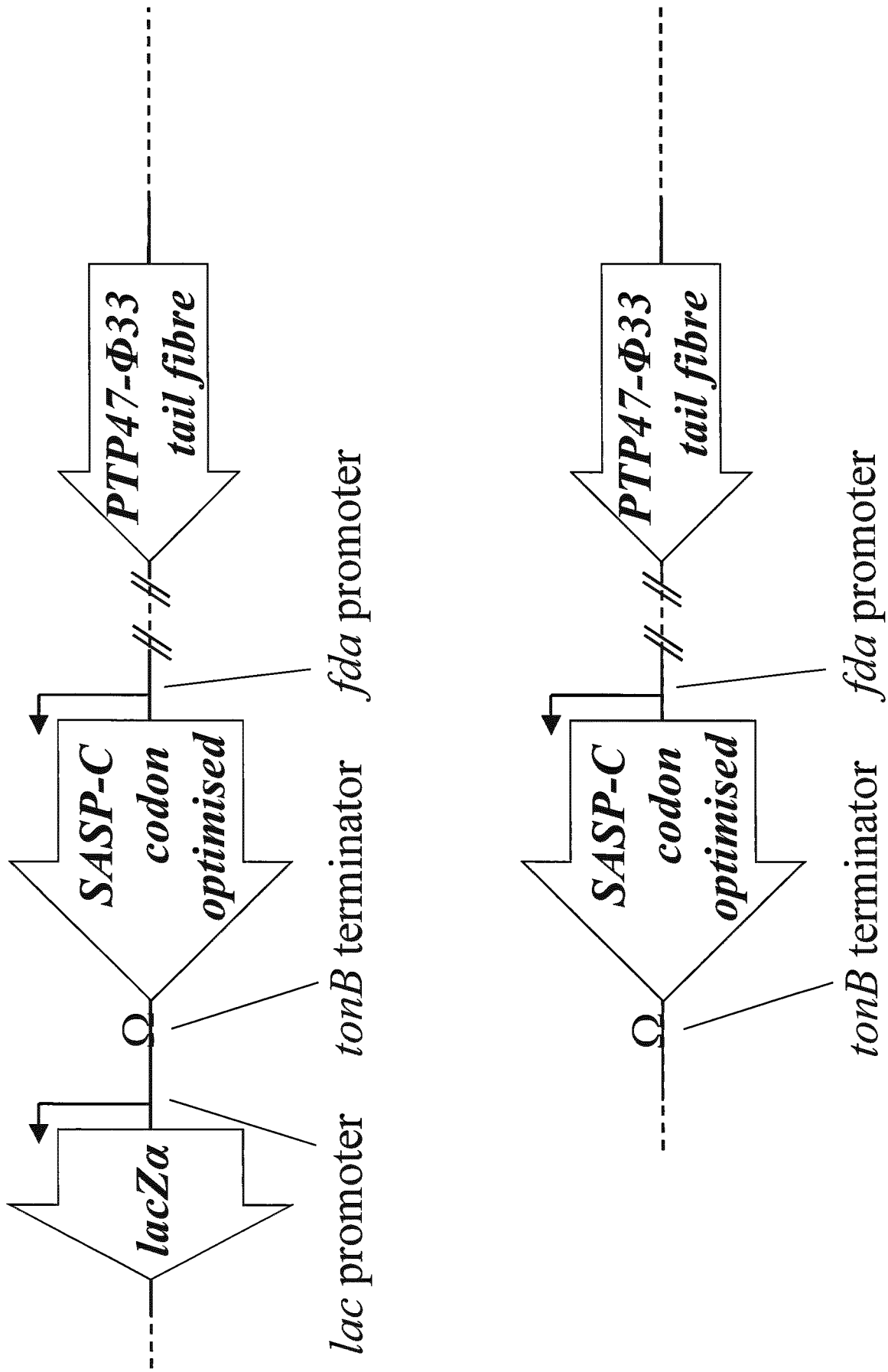


Figure 11 (Part 4 of 4)

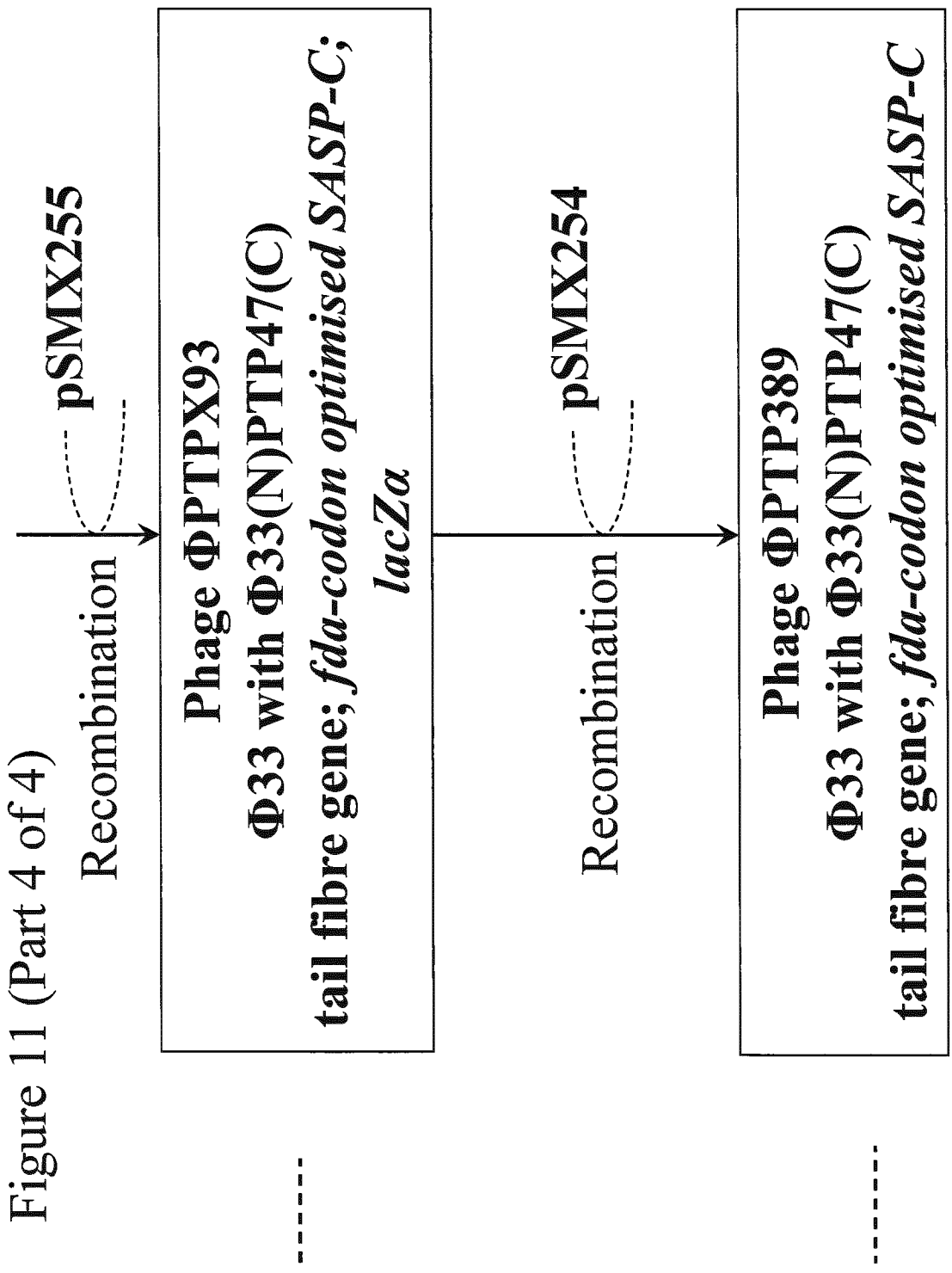


Figure 12 (Part 1 of 2)

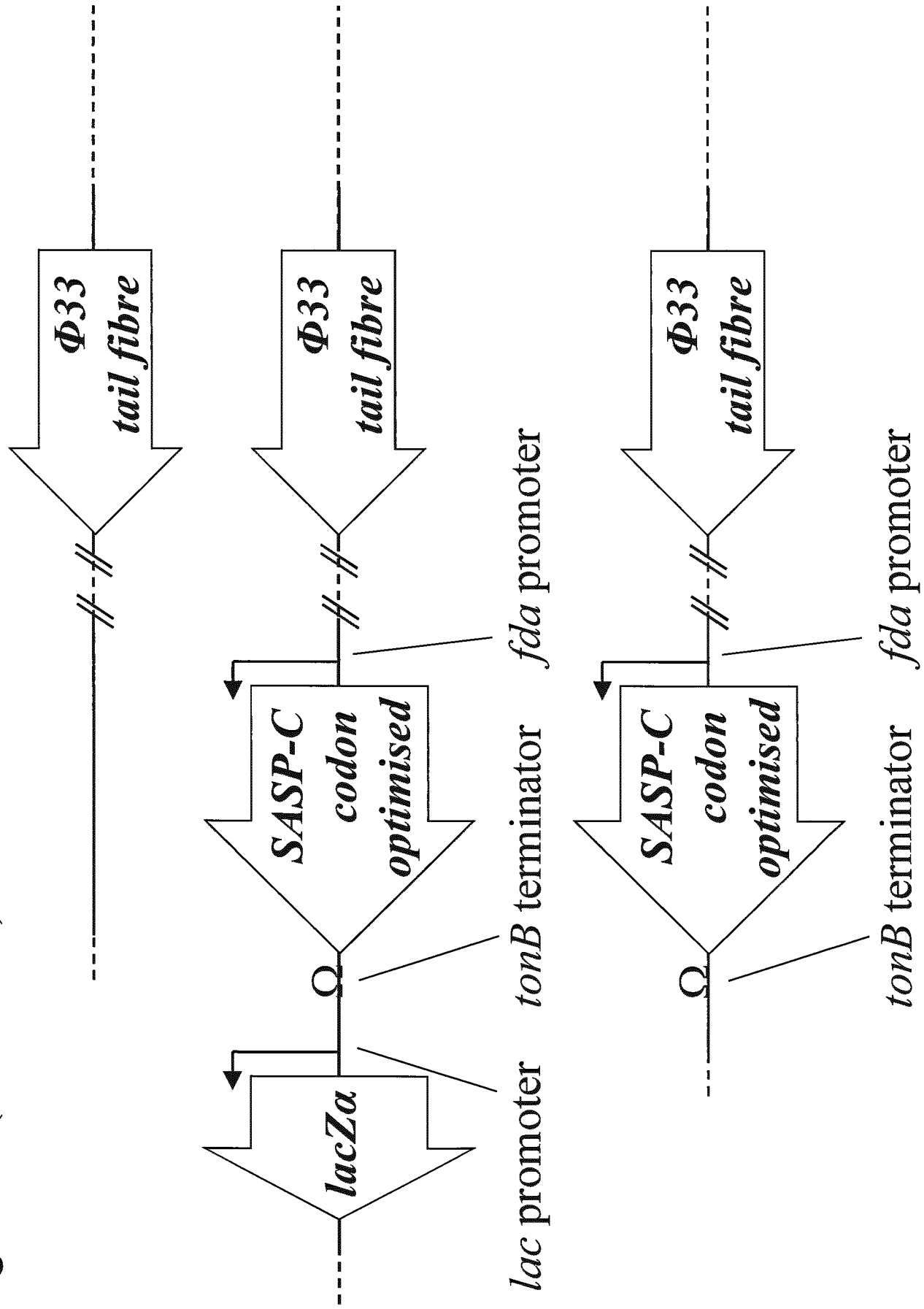


Figure 12 (Part 2 of 2)

Figure 13

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SPM-1           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
F8              MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
PB1             MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
C36             MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
LBL3            MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
Phi33           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
LMA2            MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKVVERKIQNQ 60
KPP12           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
JG024           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
PTP92           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
NH-4            MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
14-1            MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
PTP47           MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
SN              MITPELIPSPFAAQGDKDPIPQTSSTGFANLRDGYTPDYEISLASNNPQAKAVERKIQNQ 60
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SPM-1           LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
F8              LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
PB1             LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
C36             LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
LBL3            LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
Phi33           LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANAIDPLSS 120
LMA2            LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
KPP12           LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
JG024           LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
PTP92           LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
NH-4            LFFIATQNAQAWQRQMAPPWFQGMPGGYERNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
14-1            LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
PTP47           LFFIATQNAQAWQRQMAPPWFQDMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
SN              LFFIATQNAQAWQRQMAPPWFQGMPGGYEQNAEVVRVGNDGIMRRYRSMVNANASDPLSS 120
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SPM-1           TTWEEQPAWSVMRSNIPMPAGGSGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVVASQNA 180
F8              TTWEEQPAWSVMRSNIPMPAGGSGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVAASQNA 180
PB1             TTWEEQPAWSVMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVVASQNA 180
C36             TTWEEQPAWSVMRSSIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVVASQNA 180
LBL3            TTWEEQPAWSVMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVIASQNA 180
Phi33           TTWEEQPAWSVMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVVASQNA 180
LMA2            TTWEEQPAWSAMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVIASQNA 180
KPP12           TTWEEQPAWSAMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVIASQNA 180
JG024           TTWEEQPAWSVMRTNIPMPAGGPGLSSGGEVITTGRNFNELLNGTWEFFSDAIVVASQNA 180
PTP92           TTWEEQPAWSVMRTNIPMPAGGPGLSSGGEVITTGRNFNELLNGTWEFFSDAIVVASQNA 180
NH-4            TTWEEQPAWSVMRTNIPMPAGGPGLSSGGEVITTGRNFNELLNGTWEFFSDAIVVASQNA 180
14-1            TTWEEQPAWSVMRSSIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVIASQNA 180
PTP47           TTWEEQPAWSAMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVIASQNA 180
SN              TTWEEQPAWSVMRSNIPMPAGGPGLSSGGEVITTGRNFNDLLNGTWEFFSDSVVVASQNA 180
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SPM-1           PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
F8              PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
PB1             PVYPASAGAAAGMLEAKSWISGSNTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
C36             PVYPASAGAAAGMLEAKSWISGSNTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
LBL3            PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
Phi33           PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
LMA2            PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
KPP12           PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVALRGLNAGAWTNWMYAVNVMAL 240
JG024           PVYPASAGAAAGMLEAKSWVSGSNTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
PTP92           PVYPASAGAAAGMLEAKSWVSGSNTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
NH-4            PVYPASAGAAAGMLEAKSWVSGSNTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
14-1            PVYPASAGAAAGMLEAKSWISRSNTFCVQRYTDRVGNVAVRGLNAGEWTNWMYAVNVMAL 240
PTP47           PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
SN              PVYPASAGAAAGMLEAKSWVSGANTFCVQRYTDRVGNVAVRGLNAGAWTNWMYAVNVMAL 240
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SPM-1           QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMVLRVKFNAMNTGASTINVSGFGSKAIV 300
F8              QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMVLRVKFNAMNTGASTINVSGFGSKAIV 300
PB1             QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTVNTGASTINVSGFGAKAIV 300
C36             QQGRVTYGVAAGPANAYTLTLVPQLQGGLVDGMILRVKFNTVNTGASTINVSGFGAKAIV 300
LBL3            QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGASTINVSGFGAKAIV 300
Phi33           QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGASTINVSGLGAKAIV 300
LMA2            QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGATTINVSGLGAKAIV 300
KPP12           QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGASTINVSGLGAKAIV 300
JG024           QHGRVTYGTAAGPANAYTLTLVPQIQGGLVDGMILRVKFNTMNTGATTINVSGLGAKAIV 300
PTP92           QHGRVTYGTAAGPANAYTLTLVPQIQGGLVDGMILRVKFNTMNTGATTINVSGLGAKAIV 300
NH-4            QHGRVTYGTAAGPANAYTLTLVPQIQGGLVDGMILRVKFNTMNTGATTINVSGLGAKAIV 300
14-1            QQGRVTYGVAAGPANAYTLTLVPQLQGGLVDGMILRVKFNTVNTGASTINVSGLGAKAIV 300
PTP47           QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGATTINVSGLGAKAIV 300
SN              QQGRVTYGVAAGSANAYTLTLVPQLQGGLVDGMILRVKFNTMNTGASTINVSGLGAKAIV 300
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SPM-1           GAANFPLTGGELGQGLIAELVFDATGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
F8              GAANFPLTGGELGQGLIAELVFDATGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
PB1             GAANFPLTGGELGQGLIAELVFDATGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
C36             GAANFPLTGGELGQGLIAELVFDATGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
LBL3            GAANFPLTGGELGQGLIAELVFDATGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
Phi33           GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
LMA2            GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
KPP12           GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
JG024           GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVE 360
PTP92           GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVE 360
NH-4            GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
14-1            GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
PTP47           GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
SN              GAANFPLTGGELGQGLIAELVFDAAGDRWRILAGAPRIQVGNADQDYQAPSWKQVKDYVA 360
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SPM-1           SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
F8              SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
PB1             SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
C36             SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
LBL3            SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
Phi33           SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
LMA2            SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
KPP12           SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNNNRAKDFDYRFISEAD 420
JG024           SQKLTEVDWTDVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
PTP92           SQKLTEVDWTDVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
NH-4            SQKLTEVDWTDVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
14-1            SQKLTEVDWTDVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
PTP47           SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
SN              SQKLTEVDWADVVNKPNVAIQDTTPWFANLELSDARPFIDFHFNSNRAKDFDYRLISEAD 420
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SPM-1           GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
F8              GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
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PB1             GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
C36             GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
LBL3            GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
Phi33           GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
LMA2            GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
KPP12           GSMAFYSRQGSAGPTQDILFSRSNVTFLQPRLDVAKNLAYIANSGSLWQNTTADQPGWKF 480
JG024           GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
PTP92           GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
NH-4            GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
14-1            GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPIWQNTTADQPGWKF 480
PTP47           GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGPLWQNTTADQPGWKF 480
SN              GSLAFYSRQGSAGPTQDILFNRNSVTFFQPRLDVAKNLAYIANSGSLWQNTTADQPGWKF 480
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SPM-1           TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
F8              TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
PB1             TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
C36             TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
LBL3            TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
Phi33           TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYVPWDSGNFD 540
LMA2            TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
KPP12           TFAQGVDANNNAVIAVNTTNPDGSYRSQIMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
JG024           TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
PTP92           TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
NH-4            TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
14-1            TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNDRPLFAGQYTPWDSGNFD 540
PTP47           TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
SN              TFAQGVDANNNAVIAVNTTNPDGSYRSQVMRWDWASTNVIFNNRPLFAGQYTPWDSGNFD 540
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SPM-1           PATKLTVGTTNNISGPTGIRNTTSNTGNMNTWGSSSTTASYGNAALQIFGRGGGEPAAIY 600
F8              PATKLTVGTTNNISGPTGIRNTTSNTGNMNTWGSSSTTASYGNAALQIFGRGGGEPAAIY 600
PB1             PATKLTVGTTNNISGPTGIRNTTSNTGNMNTWGSSSTTASYGNAALQIFGRGGGEPAAIY 600
C36             PATKLTVGTTNNISGPTGIRNTTSNTGNMNTWGSSSTTASYGNAAVQIFGRGDGEPAAIY 600
LBL3            PATKLTVGTTNNISRPTGIRNTTSNTGNMNTWGSSSTTASYGNAALQIFGRGGGEPAAIY 600
Phi33           PSTKLTVNATNQIAGPTGIRNTNGNTGNMNTWGSGSTTASYGNAALQIFGKGGGEPAALY 600
LMA2            PSTKLTVNATNQIAGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
KPP12           PSTKLTVNATNQIAGPTGIRNTNGNTGNMNTWGSSSTTASYGNAALQIFGKGGGEPAALY 600
JG024           PSTKLTVSATNQISGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
PTP92           PSTKLTVSATNQISGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
NH-4            PSTKLTVSATNQIAGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
14-1            PSTKLTVSATNQIAGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
PTP47           PSTKLTVSATNQIAGPTGIRNTNGNTGNMNTWGSGSTTASYGNAAIRIFGKGGGEPAAIY 600
SN              PSTKLTVRATNQIAGPTGIQNTNGNTGNMNTWGSGSTTASYGNAAIQIFGKGGGEPAAIY 600
                *:***** :**:*: ****:**..**********.**********::***:*.*****:* 

SPM-1           FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVVTDESNIRNHVNGMSGAPVWGGQWFWGEW 660
F8              FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVVTDESNIRNHVNGMSGAPVWGGQWFWGEW 660
PB1             FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVVTDESNIRNHVNGMSGAPVWGGQWFWGEW 660
C36             FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVVTDESNIRFHVNSMAGTPVWGGNEFWGPW 660
LBL3            FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVVTDESNIRFHVNSMAGTPVWGGNEFWGPW 660
Phi33           FDNSQTGWYLGMDKDGQLKRAGWSLGNNAYVITDESNIRFHVNSMAGTPVWGGNEFWGPW 660
LMA2            FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDELNIRNHINGMAARPVWGGNEFWGPW 660
KPP12           FDNSQTGWYLGMDKDGQLKRAGWSLGNNAYVITDESNIRFHVNSMAGTPVWGGNEFWGSW 660
JG024           FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGNVEFWGPW 660
PTP92           FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGNVEFWGPW 660
NH-4            FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGNVEFWGPW 660
14-1            FDNSQTGWYLGMDKDGRLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGNVEFWGPW 660
PTP47           FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGNVEFWGPW 660
SN              FDNSQTGWYLGMDKDGQLKRAGWSLGNNSYVITDESNIRTHINTMAARPIWGGVEFWGPW 660
                ****************:***********:**:*** *** *:* *:. *:**.  *** * 

SPM-1           NFNPNTKLTIKAGTQETSSTAIFSGTLPFAPIASLSDYSQAPLTIYN--SPTGPSAKPAV 718
F8              NFNPNTKLTIKAGTQETSSTAIFSGTLPFAPIASLSDYSQAPLTIYN--SPTGPSAKPAV 718
PB1             NFNPNTKLTIKAGTQETSSTAIFSGTLPFAPIASLSDYSQAPLTIYN--SPTGPSAKPAV 718
C36             NFNPNTKLTIKAGTQETSSTAIFSGTMPFAPIASLSDYSQAPLTIYN--SPTGPSAKPAV 718
LBL3            NFNPNTKLTIKAGTQETSSTAIFSGTMPFAPIASLSDYSQAPLTVYN--SPTGPSAKPAV 718
Phi33           NFNPNTKLTIKAGTQETSSTAIFSGTMPFAPIASLSDYSQAPLTVYN--APTGPSAKPAV 718
```

```
LMA2            NFNPNTKLTIKAGTQETSSTAIYSGTMPFAPIASLSDYSQAPLTIYN--APTGPSAKPAV 718
KPP12           NFNPNTKLTIKAGTQETSSTAIFSETMPFAPIASLSDYSQAPLTIYN--APTGPSAKPAV 718
JG024           NFDPNLKLTLNAFNDSSYTRMTNSGAKDVG-IASMTSYADAAMSFFNYEASNPTGPRAAV 719
PTP92           NFDPNLKLTLNAFNDSSYTRMTNSGAKDVG-IASMTSYADAAMSFFNYEASNPTGPRAAV 719
NH-4            NFNPNTKLTLGSFNDSQHTRMVNSAAKDVG-IASMTSYADAAMSFFNYEASTPTGNRAAV 719
14-1            NFDPNLKLTLNAFNDSSYTRMTNSGAKDVG-IASMTSYADAAMSFFNYEASNPTGPRAAV 719
PTP47           NFNPNTKLTLGSFNDGQHTRMVNSAAKDVG-IASMTSYADAAMSFFNYEASTPTGNRAAV 719
SN              NFNPNTKLTLGSFNDSQHTRMVNSAAKDVG-IASMTSYADAAMSFFNYEASTPTGNRAAV 719
                **:** ***: : .:   :    * :  .. ***::.*::*.:::.:*  :.. .. :.**

SPM-1           IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNNSREIADSSNIMNLWASNPTAPSWNGQTVW 778
F8              IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNNSREIADSSNIMNLWASNPTAPSWNGQTVW 778
PB1             IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNNSREIADSSNIMNLWASNPTAPSWNGQTVW 778
C36             IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNSSREIADSSNIMNLWAANPTAPSWNGQTVW 778
LBL3            IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNSSREIADSSNIMNLWAANPTAPTWNGQTIW 778
Phi33           IAFIRPGNWGAFFGIDTDNKLKWGGGSLGNSSREIADSSNIMNLWAANPTAPSWNGQTIW 778
LMA2            IAFIRPGNWGAFFGLDTDNKLKWGGGSLGNSSMEIADSSNIMNLWAANPTAPTWNGQTVW 778
KPP12           IAFIRPGNWGAFFGLDTDNKLKWGGGSLGNSSREIADSRNIMNLWAANPTAPTWNGQTVW 778
JG024           ISFVRNGSRGVLFGLDSDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
PTP92           ISFVRNGSRGVLFGLDSDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
NH-4            ISFVRNGARGVLFGLDTDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
14-1            ISFVRNGSRGVLFGLDSDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
PTP47           ISFVRNGARGVLFGLDTDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
SN              ISFVRNGARGVLFGLDTDNKLKWGGYSLGAVAFEIADSNNLMSLWSSHAAAPNWNGQTIW 779
                *:*:* *  *.:**:*:******** ***  : ***** *:*.**::::.**.*****:*

SPM-1           RSGNFDPATKVDLNAANATNGSMIFNRISGTGSGIASSGRVGAINLQNGAHSGQAAAVTF 838
F8              RSGNFDPATKVDLNAANATNGSMIFNRISGTGSGIASSGRVGAINLQNGAHSGQAAAVTF 838
PB1             RSGNFDPATKVDLNAANATNGNMIFNRISGTGSGIASSGRVGAINLQNGAHSGQAAAVTF 838
C36             RSGNFDPATKVDLNAANATNGNMVFNRISGTGSGIASSGRVGAINLQNGAHSGQAAAVTF 838
LBL3            RSGNFDPATKVDLNAANATNGNMIFNRIAGTGSGIASSDRVGAISLQNGATAGAAAAVTF 838
Phi33           RSGNFDPATKVDLNAANATNGNMIFNRIAGTGSGIASSGRVGAINLQNGEHSGQAAAVTF 838
LMA2            RSGNFDPATKVDLNAPNATNGNMIFNRIAGTGSGIASSGRVGAISLQNGATAGAAAAVTF 838
KPP12           RSGNFDPATKVDLNAPNATNGNMIFNRIAGTGSGIASSGRVGAISLQNGATAGAAAAVTF 838
JG024           RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
PTP92           RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
NH-4            RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
14-1            RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
PTP47           RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
SN              RSGNFNPDTKATLAARNTTSSPTIFS---YGASGIASTGQVGALVVENNSVTNTAAAITF 836
                *****:* **. * * *:*..  :*.     .*****:.:***: ::*.  :. ***:**

SPM-1           ERGGSIFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLGGVGSYGIF 898
F8              ERGGSIFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLGGVGSYGIF 898
PB1             ERGGSIFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLGGVGSYGIF 898
C36             ERGGSIFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLGGVGSYGIF 898
LBL3            ERGGGFFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLGGVGSYGIF 898
Phi33           ERGGSIFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNNYINQALVQVGLEGVGSYGIF 898
LMA2            ERGGGFFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNSYINQALVQVGLGGVGSYAAL 898
KPP12           ERGG-FFVNFGLDTDNVLKVGGGNLGANAYPVIHAGNYNSYINQALVQVGLGGVGSYAAL 897
JG024           HSPQKYQVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGGVGSYAIL 896
PTP92           HSPQKYQVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGGVGSYAIL 896
NH-4            HSPQKYQVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGGVGSYAIL 896
14-1            HSPQKYQVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGGVGSYGIF 896
PTP47           HSPQKYHVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGEVGSYGIF 896
SN              HSPQKYQVNFGLDADNVVKIGGGTMGGVAYPIIHSGNYNNYINQALVQVGLGGVGSYGIF 896
                .      ******:***:*:***.:*. ***:**:****.***********  ****. :

SPM-1           AVLDNAAPIATVQPGVVVDGSILIYSSCAANYNSGQKPAGTWRCMGYVVNRDANTADSAT 958
F8              AVLDNAAPIATVQPGVVVDGSILIYSSCAANYNSGQKPAGTWRCMGYVVNRDANTADSAT 958
PB1             AVLDNAAPIATVQPGVVVDGSILIYSSCAANYNSGQKPAGTWRCMGYVVNRDANTPDSAT 958
C36             AVLDNAAPIATVQPGVVVDGSILIYSSCSANYNSGQRPAGTWRCMGYVVNRDANTPDSAT 958
LBL3            AVLDYAAPTATVQPGVIVDGSILIYSSCSAHYNSGQRPAGTWRCMGYVLNRDARDPDSAT 958
Phi33           AVLDNAAPTATVQPGVVVDGSILIYSSCAANYNSGKRPAGTWRCMGYVVNRDANTPDSAT 958
LMA2            AVYDTSAPASSVGPGTILDGSVLFYSSFNANFRSGTKPTGTWRCMGYILNRDGTNPDSAT 958
KPP12           AVYDTSAPASSVGPGTILDGSVLFYSSFDANFRSGTKPTGTWRCMGYVLNRDGTNPDSAA 957
JG024           AVLDTSAPAASIAPGTIMDSSKLFYSSCDSTYRSSASPTGTWRCMGYVYNRDSTNGDSAS 956
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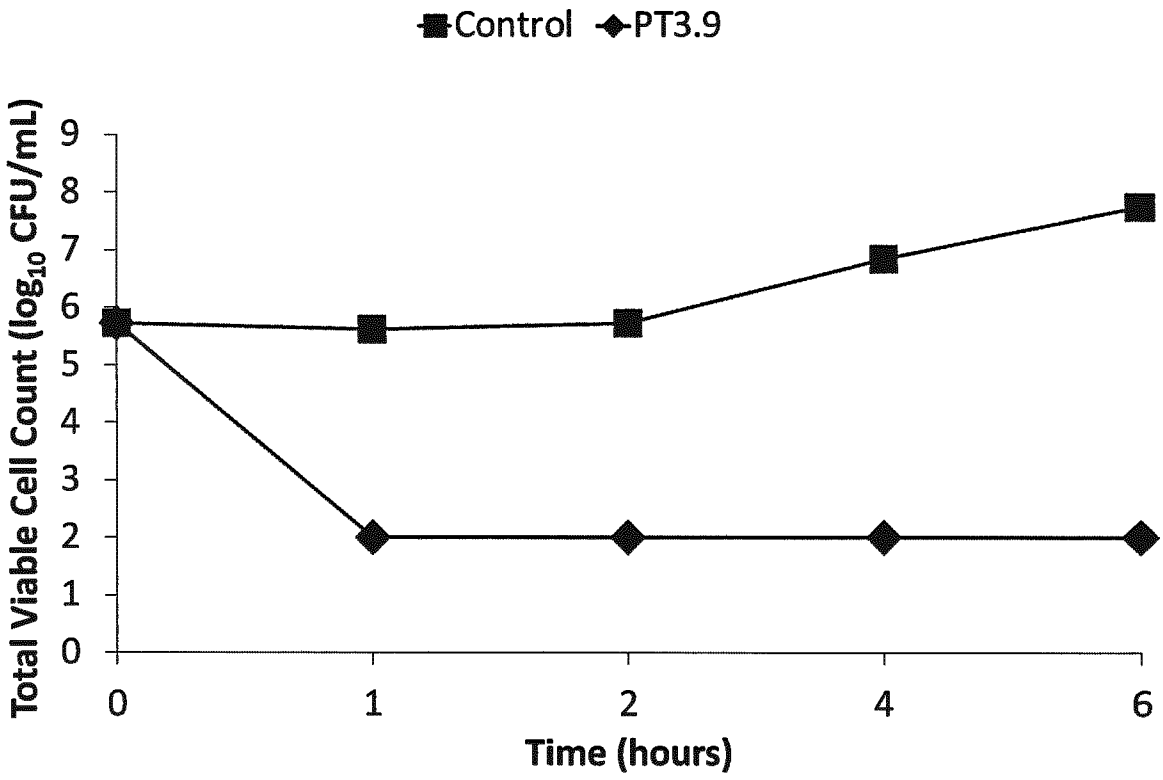
PTP92           AVLDTSAPAASIAPGTIMDSSKLFYSSCDSTYRSSASPTGTWRCMGYVYNRDSTNGDSAS 956
NH-4            AVLDTSAPAASIAPGTIMDSSKLFYSSCDSTYRSSARPTGTWRCMGYVYNRDSTNGDSAS 956
14-1            AVLDNAAPIATVQPGVVVDGSILIYSSCAANYNSGQRPAGTWRCMGYVVNRDANTPDSAT 956
PTP47           AVLDYAAPTATVRPGVVVDGSILIYSSCAANYNSGQRPAGTWRCMGYVVNRDANTPDSAT 956
SN              AVLDNAAPTATVQPGVVVDGSILIYSSCAANYNSGQRPAGTWRCMGYVVNRDANTPDSAT 956
                ** * :** ::: **.::*.* *:***  : :.*.  *:********: ***.   ***:

SPM-1           LFQRVT 964 (SEQ ID NO: 45)
F8              LFQRVT 964 (SEQ ID NO: 46)
PB1             LFQRVT 964 (SEQ ID NO: 47)
C36             LFQRVT 964 (SEQ ID NO: 48)
LBL3            LFQRVT 964 (SEQ ID NO: 49)
Phi33           LFQRVT 964 (SEQ ID NO: 50)
LMA2            LFQRVT 964 (SEQ ID NO: 51)
KPP12           LFQRVT 963 (SEQ ID NO: 52)
JG024           LFQRVT 962 (SEQ ID NO: 53)
PTP92           LFQRVT 962 (SEQ ID NO: 54)
NH-4            LFQRVT 962 (SEQ ID NO: 55)
14-1            LFQRVT 962 (SEQ ID NO: 56)
PTP47           LFQRVT 962 (SEQ ID NO: 57)
SN              LFQRVT 962 (SEQ ID NO: 58)
```

Figure 14

(A)


Control
PT3.9
Total Viable Cell Count (log_{10} CFU/mL)
0
1
2
3
4
5
6
7
8
9
0
1
2
4
6
Time (hours)

(B)

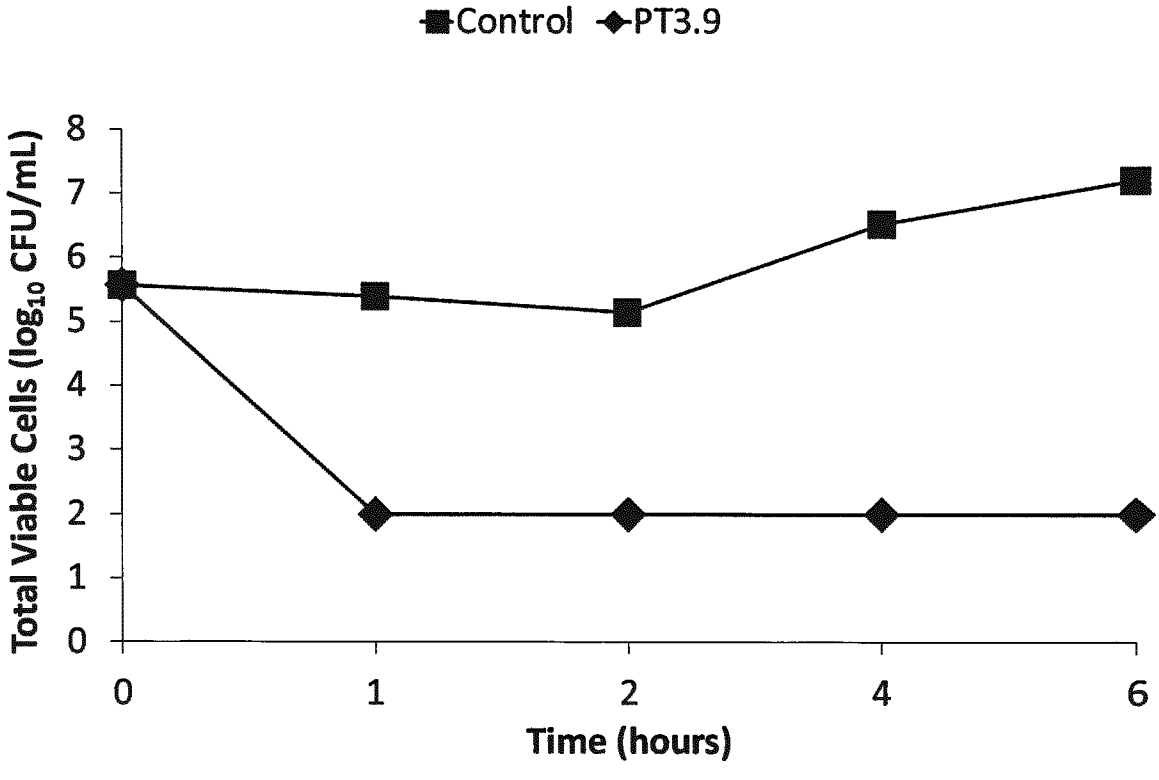
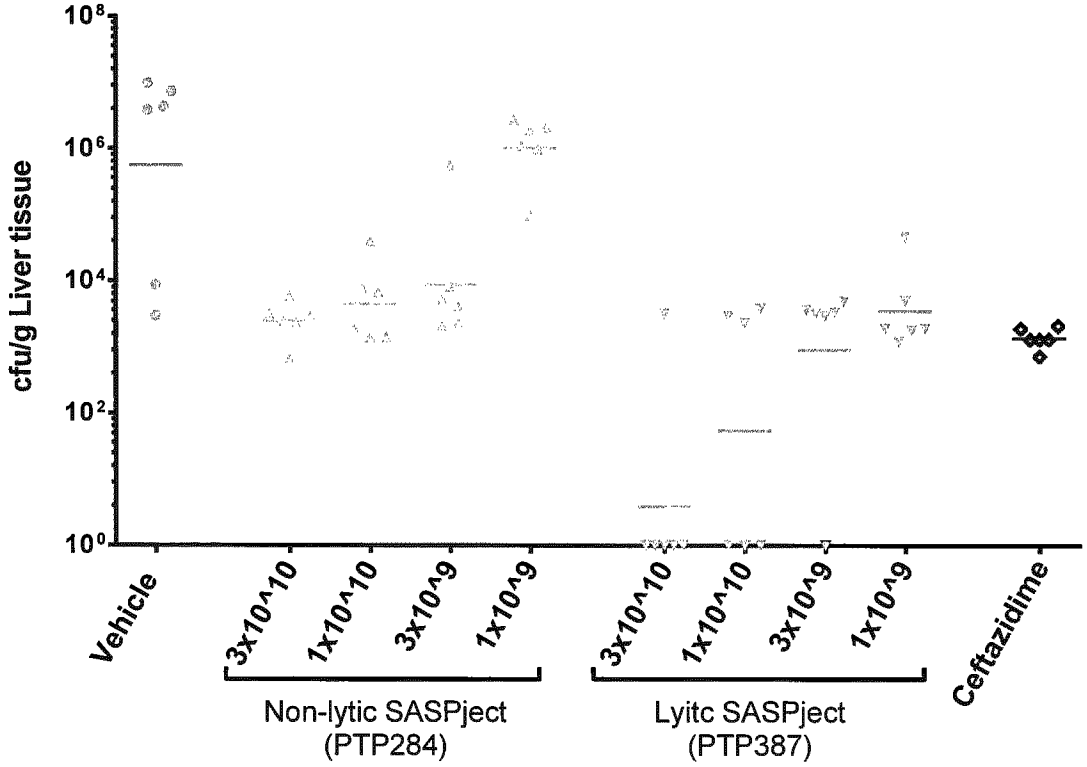

Control
PT3.9
Total Viable Cells (log_{10} CFU/mL)
0
1
2
3
4
5
6
7
8
0
1
2
4
6
Time (hours)

Figure 15


cfu/g Liver tissue
10^8
10^6
10^4
10^2
10^0
Vehicle
3x10^10
1x10^10
3x10^9
1x10^9
3x10^10
1x10^10
3x10^9
1x10^9
Ceftazidime
Non-lytic SASPject
(PTP284)
Lyitc SASPject
(PTP387)

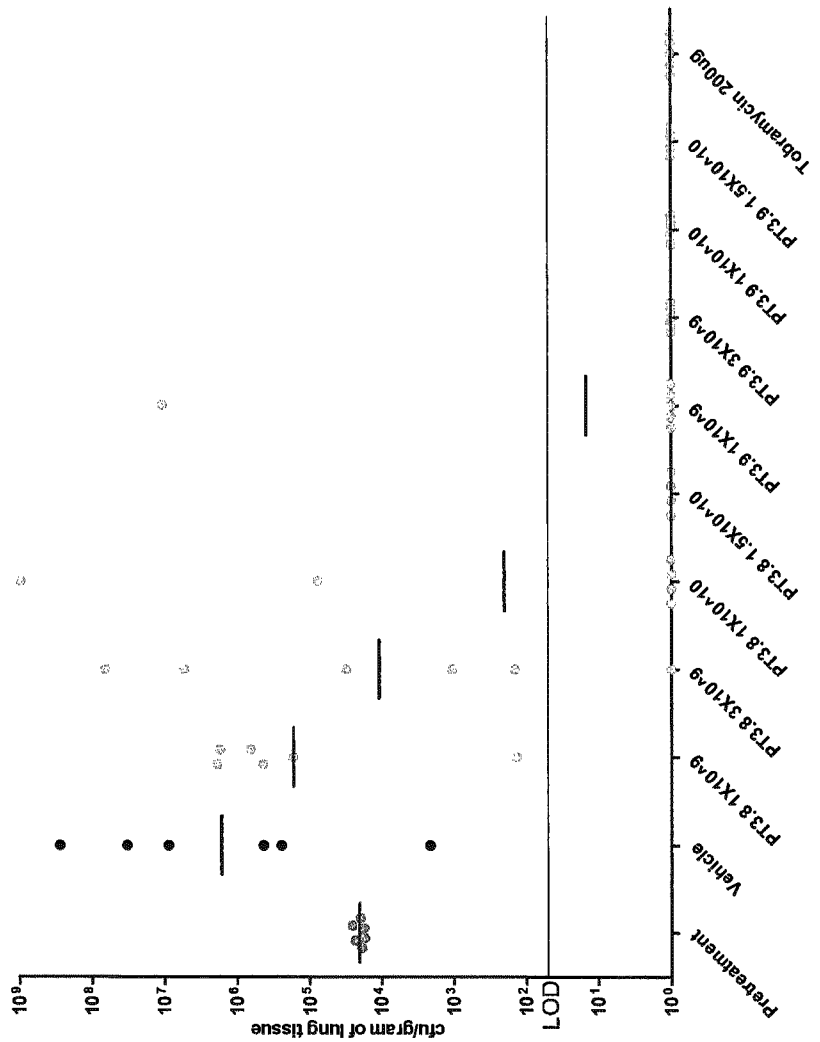

cfu/gram of lung tissue
LOD
Pretreatment
Vehicle
PT3.8 1X10^9
PT3.8 3X10^9
PT3.8 1X10^10
PT3.8 1.5X10^10
PT3.9 1X10^9
PT3.9 3X10^9
PT3.9 1X10^10
PT3.9 1.5X10^10
Tobramycin 200ug

Figure 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/058470

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 C12N15/74
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PINGFENG YU ET AL: "Isolation of Polyvalent Bacteriophages by Sequential Multiple-Host Approaches", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 82, no. 3, 20 November 2015 (2015-11-20), pages 808-815, XP055382161, ISSN: 0099-2240, DOI: 10.1128/AEM.02382-15 tables 3, 4 -----	1,5, 9-11, 13-15,28
X	WO 2009/019293 A1 (PHICO THERAPEUTICS LTD [GB]; FAIRHEAD HEATHER [GB]; WILKINSON ADAM [GB]) 12 February 2009 (2009-02-12) page 4, paragraph 2 - page 5, paragraph 2; claims 1-18 page 16, paragraph 5 page 6, paragraph 3 - page 8, paragraph 1 ----- -/--	1-7,13, 14,28, 32-39, 41-45

[X] Further documents are listed in the continuation of Box C.

[X] 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

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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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 2017	27/06/2017

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

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/058470

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/113375 A2 (PHICO THERAPEUTICS LTD [GB]; FAIRHEAD HEATHER [GB]) 29 December 2004 (2004-12-29) claims 11-34 -----	1-5,9, 10, 13-15, 28, 31-39, 41-45
X,P	WO 2016/055584 A1 (PHICO THERAPEUTICS LTD [GB]) 14 April 2016 (2016-04-14) claims 1-37 -----	1-45

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/058470

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		US 2016319244 A1	03-11-2016
		WO 2009019293 A1	12-02-2009
WO 2004113375 A2	29-12-2004	NONE	
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		WO 2016055584 A1	14-04-2016