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Applying antibiotic selection markers for nematode genetics

Eric Cornes [a,b,c], Cécile A.L. Quéré [b,c], Rosina Giordano-Santini [d], Denis Dupuy [b,c,*]

[a] Cancer and Human Molecular Genetics, Bellvitge Biomedical Research Institute-IDIBELL, Hospitalet de Llobregat, Barcelona 08908, Spain
[b] Univ. Bordeaux, IECB, Laboratoire ARNA, F-33600 Pessac, France
[c] INSERM, U869, Laboratoire ARNA, F-33000 Bordeaux, France
[d] Molecular and Cellular Neurobiology Laboratory, The University of Queensland, Queensland Brain Institute, Qld 4072, Australia

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ABSTRACT

Antibiotic selection markers have been recently developed in the multicellular model organism *Caenorhabditis elegans* and other related nematode species, opening great opportunities in the field of nematode transgenesis. Here we describe how these antibiotic selection systems can be easily combined with many well-established genetic approaches to study gene function, improving time- and cost-effectiveness of the nematode genetic toolbox.

1. Introduction

For more than forty years, antibiotic resistance genes have been used in combination with antibiotics as selectable markers for the efficient monitoring of DNA transformation in bacteria [1,2]. Due to its versatility and numerous advantages, this system has been rapidly adapted to other research models, like yeast and cultured eukaryotic cells [3]. However, applications in multicellular organisms such as *Cænorhabditis elegans* and related nematodes have not been developed until very recently [4–6].

DNA transformation of *C. elegans* was successfully developed in the 80s [7,8], and since then it has become an invaluable tool, widely applied to functional studies in nematodes. The many applications of *C. elegans* transgenesis to the study of gene function have been extensively reviewed elsewhere [9].

1.1. C. elegans transgenesis

The principle of DNA transformation in *C. elegans* relies on the introduction of exogenous DNA (plasmid or PCR product) directly into the syncytial gonad of the self-fertilizing hermaphrodite, either by DNA microinjection or by particle bombardment.

DNA microinjection is a relatively easy technique, which results in the formation of extrachromosomal arrays, consisting of multiple copies (80–300) of the exogenous DNA arranged as concatemers [7,10]. These arrays behave as artificial chromosomes, as they are efficiently replicated and segregated to the progeny, producing stable transgenic lines [7,10]. However, extrachromosomal arrays can be lost during cell divisions, leading to a variable transmission rate (depending on the strain, the array is transmitted from 10% to 90% of the progeny) [10].

During gene bombardment, DNA-coated beads are used as vectors to introduce DNA into the animals [11]. This method also produces extrachromosomal arrays, but in addition, random integration of several copies of the transgene into the genome is observed in 1/4 to 1/8 of the obtained strains [12,13]. Nowadays, the most established bombardment protocol uses *unc-119(ed3)* mutant animals as a recipient strain. These animals display an easy observable locomotor defect and are unable to enter the diapause state *Dauer* upon starvation [14]. During bombardment, the *unc-119* gene is used as a co-transformation marker, allowing the selection of transformed animals based on their wild-type locomotion and their ability to survive starvation [14].

The majority of transformation markers used for *C. elegans* transgenesis are easily scorable under a dissecting scope. They are based on the rescue of nonlethal mutations [12,15], and the use of dominant [8,10] or fluorescent markers [16,17], allowing visual identification of specific traits (Table 1). In most cases, these

Abbreviations: PCR, polymerase chain reaction; GFP, green fluorescent protein; RNAi, RNA interference; MosSCI, Mos mediated single copy insertion; NGM, nematode growth medium; NeoR, neomycin resistance gene; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; TALENs, Transcription Activator-Like Effector Nucleases; *Dpy*, dumpy; *Unc*, uncoordinated; *Lin*, cell lineage variant.

* Corresponding author at: Univ. Bordeaux, IECB, Laboratoire ARNA, F-33600 Pessac, France.
E-mail address: d.dupuy@iecb.u-bordeaux.fr (D. Dupuy).

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Table 1
Available markers for *C. elegans* transgenesis. Mutant rescue: the introduction of the wild type gene rescues a mutant phenotype. *sup-7(st-5)* rescues temperature sensitive sterility of *tra-3(e1107)* mutants [8], *lin-15(+)* rescues temperature sensitive multivulva phenotype of *lin-15(n765ts)* [36], *dpy-20(+)* rescues *dpy-20(e1282ts)* dumpy phenotype [37], *unc-119(+)* rescues *unc-119(ed3)* locomotor and *Dauer* larva formation defects [14], *pha-1(+)* rescues embryonic lethality of *pha-1(e2123)* at 25 °C [15]. Dominant phenotypes: *rol-6(su1006)* gives a roller phenotype [38], *unc-22* antisense gives a twitcher phenotype [39]. Fluorescent reporters: fluorescent proteins such as GFP (green fluorescent protein), mCherry or dsRed (red fluorescent proteins) expressed under the control of a strong promoter. Antibiotic selection: a resistance cassette gives a selective advantage to transgenic individuals in the presence of antibiotics: NeoR: G418 [5]; PuroR: puromycin [4]; HygR: hygromycin B [6]. Compatible with any genotype: the marker does not require a specific genetic background to be efficient and can be used directly with any recipient strain. Hands off selection: the marker confers a selective advantage for transformed versus non-transformed animals (*only in specific environmental conditions). Compatible with other nematode species: fluorescent and antibiotic markers can be directly expressed in other nematode species under the control of *C. elegans* regulatory sequences.

	Compatible with any genotype	Hands-off selection	Compatible with other nematodes
Mutant rescue			
dpy-20(+)	✗	✗	✗
sup-7(st5)	✗	✗	✗
lin-15(+)	✗	✓*	✗
pha-1(+)	✗	✓*	✗
unc-119(+)	✗	✓*	✗
Dominant phenotypes			
unc-22 antisense	✓	✗	✗
rol-6(su1006)	✓	✗	✗
Fluorescent markers	✓	✗	✓
Antibiotic selection			
NeoR	✓	✓	✓
PuroR	✓	✓	✓
HygR	✓	✓	✓

markers do not provide a selective advantage to transformed animals compared to the non-transformed siblings, and when they do, it is only in specific non-permissive conditions (i.e., starvation for *unc-119(ed3)* mutants). Moreover, these markers require the use of specific mutant animals as recipient strains, which are generally more difficult to grow than the wild type, and may complicate genetic interaction studies.

With some transformation markers the selection and manual maintenance of non-integrated transgenic strains (extrachromosomal array lines) is a time consuming task. Based on visual marker scoring, transgenic worms need to be selected and transferred from one plate to another every few generations. This process has to be performed repeatedly if transgenic populations have to be enriched for biological analysis or maintained for long periods of time.

1.2. Antibiotic markers for nematode transgenesis

Antibiotic selection in nematodes is based on the transformation of animals with vectors carrying a bacterial antibiotic resistance cassette, expressed under the control of a nematode ubiquitous promoter. Three distinct markers have been published to date, based on neomycin [5], puromycin [4] and hygromycin B [6,18] resistance, which offer the possibility to generate antibiotic resistant animals both by microinjection and microparticle bombardment.

Antibiotic resistance cassettes are universal markers that can be used in many different nematode species and in any genetic background, facilitating comparative evolutionary and genetic studies in nematode models. Successfully transformed animals can develop and reproduce normally in solid or liquid antibiotic containing media, whereas non-transformed siblings arrest at early larval stages. Antibiotic resistance markers thus allow both hands-off obtention and maintenance of transgenic populations [4,5]. Since almost 100% of the animals growing on selective medium are transgenic, independently of the array transmission rate, non-integrated strains can be maintained without human intervention [4,5]. Thus basic routine techniques such as strain freezing or synchronization, as well as biological analyses where a large number of animals are needed can be performed without manual selection of transgenic animals.

A common concern among the community is the expensiveness of the use of antibiotics. Based on the online catalog of Fisher Scientific 2013, we calculated that the cost for one 9 cm plate of NGM is around 0.067$ and is increased to 0.27$ by G418, and to 0.11$ by hygromycin-B. In other words, G418 and hygromycin B increase the cost by less than 15$/L of NGM (Puromycin is 10 times more expensive). However, these costs may vary depending on supplier and country. In our experience, the time saved expressed in person.hour largely outweighs the extra cost of the antibiotics themselves.

Moreover, working with antibiotics as transformation markers does not imply a constant use of selective plates for maintenance. Extrachromosomal arrays can be integrated in the genome by different approaches (see Section 3), thus eliminating the need for further antibiotic selection. The extra cost of antibiotics can then be limited to the selection of the desired integrated transgenic strains, making it an economically viable and cost effective method affordable by all *C. elegans* laboratories.

2. Materials and methods

Several vectors containing a NeoR cassette were developed in our laboratory [5] (available at https://www.addgene.org/Denis_Dupuy/). We routinely use NeoR as a co-injection marker to easily and rapidly generate non-integrated strains by microinjection. We provide here our protocol with all the information required to efficiently generate and maintain transgenic lines carrying extrachromosomal arrays.

2.1. G418 plate preparation

NGM-G418 plates are prepared as standard NGM plates (3 g NaCl, 12 g agar, 2.5 g peptone, 975 ml H2O; adding 1 ml cholesterol (5 mg/ml), 1 ml 1 M $MgCl_2$, 1 ml 1 M $CaCl_2$ and 25 ml 1 M potassium phosphate (KPO_4) buffer after autoclave). G418 (G418 Sulfate dissolved in sterile water) is added at a final concentration of 0.4 mg ml^{-1} immediately prior to pouring plates to avoid thermo degradation. All reagents were purchased from Fisher Scientific (G418 sulfate: BP673-5). Plates are left to dry at room temperature (20–22 °C) for 24 h before seeding with bacteria. OP50NeoR (*Escherichia coli* OP50 transformed with pETMCN-EK (derived from pET-28b: Ori colE1, KanR) Kan-resistant plasmid. Resistant to Kanamycin, Neomycin, G418) cultures are grown in LB (Luria Broth) media with Kanamycin (50 μg ml^{-1}) overnight at 37 °C before seeding the plates. Alternatively, the G418 solution can be spread on regular NGM plates to the same final concentration.

2.2. Storage

Plates can be stored unseeded at 4 °C for at least 2 months without detecting changes in their selectivity. Once seeded with OP50NeoR, they can be stored for at least three weeks at 4 °C. It is important to test antibiotic selectivity for every new batch just by scoring larval development of N2 animals. Pick five to ten N2 gravid adults on an antibiotic plate and score the progeny for larval arrest (no individuals should be able to grow) [5]. G418 plates are generally less prone to bacterial contamination than normal NGM plates, however some contaminant bacteria can still appear sporadically. Contaminated plates are a signal of antibiotic degradation (either prior to the contamination or due to the detoxification of the plate by an antibiotic resistant bacteria); in that case plates should be discarded.

2.3. Creating transgenic lines by DNA microinjection

For microinjection, DNA plasmids are purified using Qiaprep Spin Miniprep kit (QIAGEN). The final concentration of DNA to inject should range between 50–100 ng/μl, including a NeoR plasmid as co-injection marker at 10 ng/μl.

Microinjection is performed as previously described [10]. Between 10 and 20 injected hermaphrodites are transferred onto 6 cm NGM-G418 plates and incubated at 15–20 °C. A plate containing non-injected control animals should also be used at this step as a control for antibiotic selectivity. Developing F_1 individuals resulting from these plates should be singled out and transferred onto 3.5 cm NGM-G418 plates, according to the targeted number of independent lines. Transient transgenic animals (animals that do not pass on the array to their offspring) will not have viable progeny, therefore a cursory look at the plates after a few days is sufficient to identify stable independent lines.

2.4. Maintenance on G418

Routine methods used for *C. elegans* culture do not require any adaptation. A stable NeoR line growing on NGM-G418 plates can simply be maintained in culture by transferring a few animals either with a worm-pick, or with a chunk of G418-NGM agar from a crowded plate to a new one. Depending on the transmission rate, antibiotic resistant transgenic lines may expand slower and do not starve as quickly as with traditional markers that are rapidly overpopulated by non-transgenic animals.

3. Resistance markers and transgene integration

The non-Mendelian transmission of extrachromosomal arrays is circumvented by the use of antibiotic resistant markers, however some other genetic issues can still be associated with the structure of these arrays. High copy number arrays are silenced in the germline due to their repetitive pattern [19,20]. Multiple copies of an endogenous promoter can also cause deleterious phenotypes through transcription factor titration [21,22]. When physiological expression levels are required, it is necessary to isolate transgenic animals carrying a low copy number of the transgene integrated in the genome. It is possible to integrate extrachromosomal arrays through irradiation by X-rays, gamma rays or UV [23]. However, while this allows Mendelian transmission of the transgene, it does not affect issues related to copy numbers. The mutagenic irradiation sources also cause undesired genomic DNA damage, resulting in the accumulation of unintended mutations. The presence of these mutations requires several backcrossing steps to avoid genetic aberrations that could alter the physiological function of the pathway studied. As previously mentioned, biolistic transformation can also generate random integration of the exogenous DNA in a low copy number; supposedly with less mutagenic off target effects than the irradiation techniques.

4. Advantages of antibiotic markers for genetic approaches

4.1. Advantages of antibiotic markers for genome editing

It is only recently that methods allowing for single copy integration of transgenes in a targeted locus were developed. These methods are based on the introduction of the necessary genetic material in animals by direct microinjection in the gonadal syncytium. The MosSCI technique (Mos1 mediated single copy insertion) [24] relies on the use of a recipient strain carrying a single Mos1 transposon at the locus of interest [25,26]. The Drosophila Mos1 transposase mediates the specific excision of the transposon, and allows the targeted insertion of an exogenous DNA sequence. Initially developed with *unc-119* rescue as a positive selection marker, the MosSCI protocol also works with antibiotic selection [5,27] which removes the need for a prior cross of the recipient strain with the *unc-119(ed3)* mutant.

Similarly, hygromycin selection was used in another recently developed genome editing method which uses the CRISPR/Cas9 system [28,29]. Briefly, this system relies on a bacterial defense mechanism using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins to destroy foreign DNA or RNA. A specific RNA template can direct the Cas9 endonuclease to generate a double strand break at a

desired locus, which is then repaired using the provided homologous DNA, leading to site specific deletions or insertions [29]. The technique was first developed for gene disruption with visible loss-of-function alleles that cause easily identifiable phenotypes, such as *unc-119* (uncoordinated), *dpy-13* (dumpy) in association with the fluorescent protein mCherry as a transformation marker [29]. By comparison, the use of visible markers implies scoring the lines during growth, and isolating hundred(s) of potential positives among the F_1 and F_2 generations. For example, when disrupting *dpy-13*, only 1/210 isolated F_1 gave a Dpy progeny [29]. However, when the target gene was replaced by the hygromycin resistance cassette, only 30 injected animals were isolated after injection. Hygromycin solution was added on the injection plates when they reached the F_2 generation. This killed all animals but the mutant/transgenic animals carrying the hygromycin resistance, and gave 3 independent lines.

Another mechanism has also recently been adapted for genome engineering in *C. elegans*. Transcription Activator-Like Effector Nucleases (TALENs), composed of an engineered specific DNA binding domain and a cleavage domain can cut DNA and generate double strand breaks at specific sites [30]. This method has been successfully used to create substitutions [31] and a conditional knock-out of an embryonic lethal gene in wild type *C. elegans* [32]. To date, the TALENs have been used to disrupt genes leading to a strong visible phenotype such as *dpy-5* (Dpy), *lon-2* (Long), [32] *ben-1* (Benzimidazole resistant) [33] and *unc-119* in *Pristionchus pacificus* [31]. When the targeted gene is not associated with a visible phenotype, fluorescent proteins or the dominant roller marker were used as co-injection markers, followed by F_1 and F_2 genotyping to confirm the insertion [32]. But for this method, as well as the previous two described above, antibiotics selection markers should constitute the reporter of choice.

MosSCI, CRISPR-CAS9 and TALENs methods have also been shown to work in different nematode species such as *P. pacificus*, *Caenorhabditis* species 9 and *C. briggsae* [31] and are based on a first step of micro-injection. As described above, the three antibiotic systems available for nematode transformation (G418, puromycin and hygromycin B) have proven to be efficient selection markers for these genome engineering methods. Given that only transgenic animals survive in the presence of the antibiotic, screening for rare integration events is facilitated by a reduced progeny. Moreover, an integration event can be detected by using a negative selection marker [27] for the loss of the extrachromosomal array or by genotyping [34]. For all these methods, the use of antibiotic selection markers can facilitate and speed up both, the isolation of the initial transgenic lines, and the identification of the successful integration events.

Finally, antibiotic assisted genome editing applied to knock-out studies has the added advantage of automatically generating convenient genetic balancers in case of essential genes. If the homozygous knock-out is non-viable, only the heterozygous animals carrying one wild-type allele on one chromosome and the deleted allele replaced by the resistance marker on the other will be able to survive on selective plates.

4.2. Advantages of antibiotic markers for genetic crosses

The development of very precise genome editing techniques such as Mos mediated transgenesis, TALENs or the CRISPR/Cas9 system in *C. elegans* in combination with antibiotic resistance

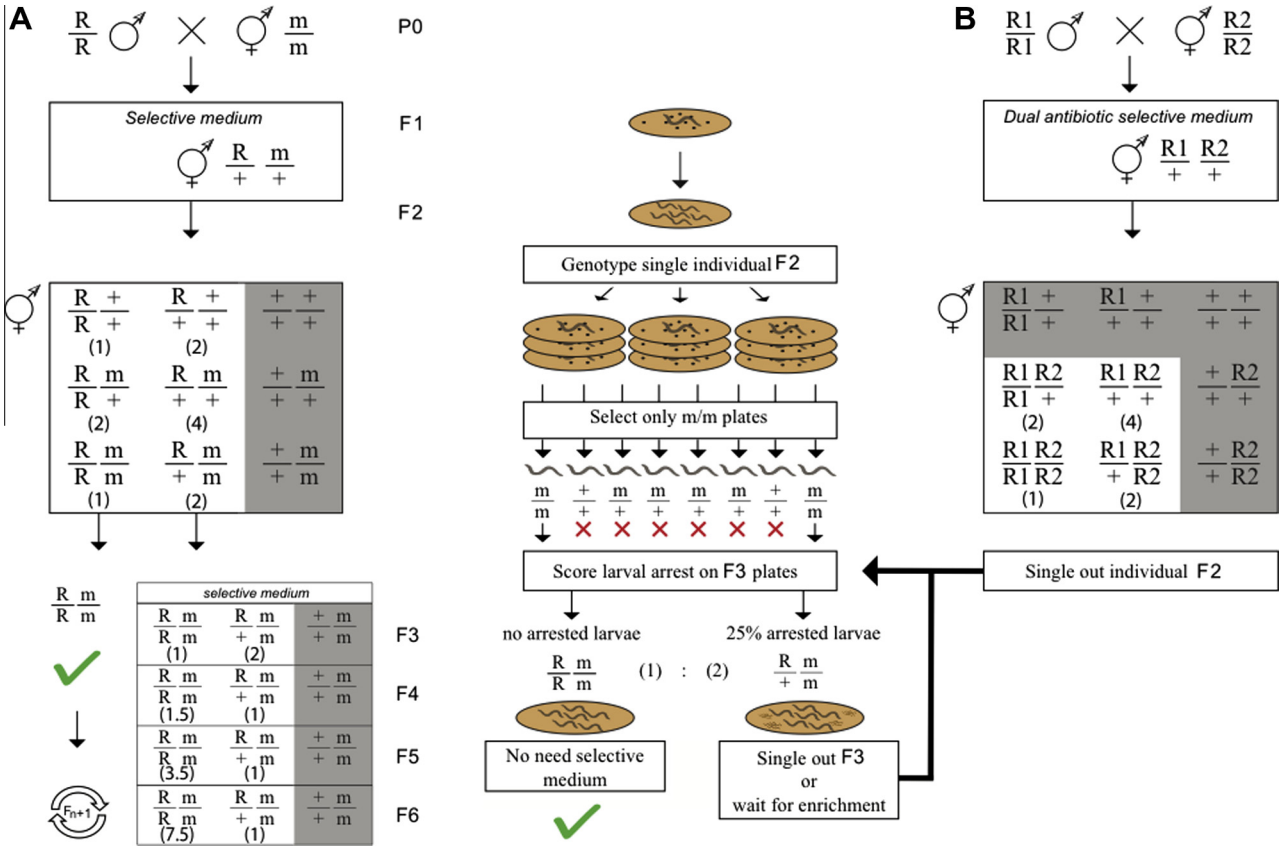

Fig. 1. Advantages of antibiotic resistance markers for genetic crosses. (A) Schematic representation of a genetic cross between an antibiotic resistant strain and a mutant strain. (B) Schematic representation of a genetic cross between two strains carrying different antibiotic markers. Dual antibiotic selection medium automatically selects for animals containing both markers. R: transgenic antibiotic marker, m: mutant allele, +: wild type allele. Shaded cells indicate animals unable to develop on the selective medium. Numbers in parenthesis indicate the proportions within the surviving population.

markers offers the possibility to insert antibiotic resistance genes in close proximity of detrimental mutant alleles. Such traits may therefore be easily enriched over wild type traits using selective plates.

One of the convenient features of *C. elegans* for genetics studies is its dual mode of reproduction. Two separated sexes, self-fertilizing hermaphrodites and males, offer the possibility of conveniently obtaining and maintaining homozygous strains by either self-fertilization or combine genotypes by cross-fertilization. More specifically, cross-fertilization strategies can be designed to generate new strains containing different combinations of mutations and/or markers, to uncover meaningful genetic interactions.

However, before the development of antibiotic resistance systems in nematodes, none of the available markers provided a real selective advantage that could be applied for genetic crossing designs. As a result, the scoring of the traits of interest had to be followed every generation by phenotyping and/or genotyping individual animals. Antibiotic selection can also be used to facilitate this process. Integrated transgenic strains containing an antibiotic marker can be crossed with any other strain, and the selective medium will automatically eliminate the offspring that do not carry the resistance marker.

For example, an integrated transgenic strain carrying an antibiotic resistance cassette (R) as co-transformation marker can be easily crossed with any viable mutant (m) strain. If the antibiotic resistance (R) is carried by the males, starting the mating step in selective medium will prevent the development of F_1 progeny coming from self-fertilization. Therefore, all the resulting F_1 hermaphrodites will be double heterozygotes. F_1 animals can be singled out at larval stage L4 and transferred onto antibiotic plates for self-fertilization. F_2 individuals are singled out again and allowed to breed before being genotyped to identify homozygote m/m plates.

–In 1/3 of those plates we will not observe antibiotic-induced larval arrest, which indicates that the F_2 progenitor carried two copies or the resistance marker (Fig. 1A). All F_3 are (m/m; R/R) homozygotes. In that case no more antibiotic selection is needed and the obtained strain can be frozen or maintained in regular NGM plates.

–In 2/3 of the cases we will observe 25% of antibiotic-induced arrested larvae, which characterize the progeny of an R heterozygote animal. At this point F_3 individuals can be singled onto new plates repeating the previous step. Alternatively, they can be left to proliferate in the selective medium for a few generations before repeating the isolation step. Thanks to the selective pressure of the antibiotic, the population will become more enriched in double homozygote animals at each generation, increasing the probability to single out the desired phenotype over time (Fig. 1A).

The selective advantage provided by the antibiotic resistance markers eliminates the need to genotype the alleles associated to the resistance marker. After genotyping the mutation m, the plates carrying antibiotic resistant animals will progressively become enriched in double homozygote animals, independently of the parental genotype (R/+;m/m or R/R;m/m), without human intervention.

Interestingly, dual antibiotic selection has been proven to efficiently isolate transgenic individuals after microparticle bombardment [35]. Combining genome editing techniques with dual antibiotic selection will allow the insertion of different antibiotic resistance markers (R1 and R2) close to different features such as alleles of interest, or fluorescent markers (Fig. 1B). Transgenic R1 males can be crossed with R2 hermaphrodites onto dual antibiotic selection plates. As described above, F_1 progeny should be singled on selective plates for self-fertilization. Any F_2 growing on a dual antibiotic plate will contain at least one copy of R1 and R2. F_2 animals can be singled out onto dual antibiotic plates and their progeny scored for the absence of larval arrest, to identify R1R2 homozygotes. Heterozygous progeny can also be maintained for a few generations on selective plates to allow for the passive enrichment of double homozygote R1 R2 animals (Fig. 1B).

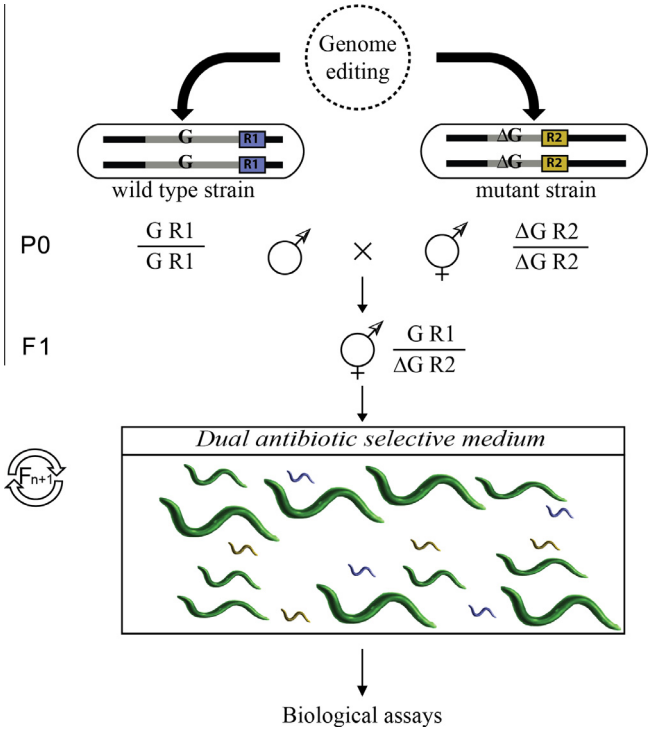

Fig. 2. Application of integrated antibiotic resistance to the study of haploinsufficiency. Schematic representation of a genetic cross between two strains carrying a different antibiotic resistance marker associated to different alleles of the same gene. Only heterozygote individuals (green) are able to develop in dual antibiotic selection medium, any homozygote individual (blue or yellow) arrests in early larval stages. R1, R2: transgenic antibiotic markers, G: wild-type allele, ΔG: mutant allele of interest.

Combination of antibiotic markers and genome editing techniques can also be useful to target a genetic locus of interest and design forced heterozygosity as follows: an antibiotic resistance cassette (R2) can be designed and integrated in a chromosome region to generate an allele (ΔG). In parallel, another antibiotic resistance gene (R1) can be introduced in the same region near the wild type copy of the same gene (G) in a wild type animal. When successfully generated, the two strains will contain an antibiotic marker near a different allele of the same gene of interest. Individuals resulting from the genetic crossing between the two strains and developing in a dual antibiotic selective medium will be forced heterozygotes for the gene of interest. The new strain can be easily maintained as a heterozygous population as long as needed using a dual antibiotic selection medium to study for example the effect of haploinsufficiency (Fig. 2).

5. Concluding remarks

Since its recent successful adaptation to nematodes, the use of single or combined antibiotic resistance markers have proven to be a very effective and efficient way to generate transgenic strains. The selective advantage offered by antibiotic resistance has already been applied to the emerging genome editing methods, demonstrating its potential for also facilitating more classical *C. elegans* genetic manipulations such as crosses.

The versatility of this system offers an attractive picture of how genetic studies can be made more convenient for modern biologists in the field. The existence of several different resistance cassettes for nematodes already allows their use in combination. Altogether, these developments will permit *C. elegans* researchers to be more selective with their markers and less picky with their worms.

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