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Cytosine Deaminase as a Negative Selection Marker for Gene Disruption and Replacement in the Genus *Streptomyces* and Other Actinobacteria▽†

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We developed a novel negative selection system for actinobacteria based on cytosine deaminase (CodA). We constructed vectors that include a synthetic gene encoding the CodA protein from *Escherichia coli* optimized for expression in *Streptomyces* species. Gene disruption and the introduction of an unmarked in-frame deletion were successfully achieved with these vectors.

Cytosine deaminase (EC 3.5.4.1), widely distributed in bacteria and fungi, catalyzes the deamination of cytosine to uracil as a part of the pyrimidine salvage pathway (20, 22). This enzyme also converts 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), a highly toxic compound. This suicide effect has been exploited in negative selection procedures for eucaryotic organisms (2, 12, 20, 26).

Counterselection markers are valuable tools facilitating gene disruption based on homologous recombination. The choice of such markers is, however, limited for streptomycetes. The glucokinase gene (*glkA*) of *Streptomyces coelicolor* A3(2) can be subjected to counterselection by using resistance to 2-deoxyglucose (8). This selection must be performed with strains in which the endogenous *glkA* gene has been deleted (8, 16). The *rpsL* gene (encoding the ribosomal protein S12) confers a dominant streptomycin-sensitive phenotype in a streptomycin resistance background, the latter being obtained by spontaneous mutations of the *rpsL* gene (4, 14, 18, 23). Such procedures, requiring the use of specifically mutated host strains, constitute a serious limitation for many studies. So far, most gene disruption procedures include a laborious colony replication step to obtain isolates that have lost an antibiotic resistance marker. This step persists even in the recent PCR targeting procedures (10, 11).

A previous study of pyrimidine salvage pathways in *Streptomyces* (15) noted the absence of cytosine deaminase. Accordingly, there are no apparent *codA* orthologs in genomic sequences of most actinobacteria (R. Brzezinski, unpublished data). Therefore, it would be possible to use the CodA-based counterselection procedure for streptomycetes without any genetic modification of the host strain. We report here new vectors for gene disruption and replacement based on this principle.

Plasmids, strains, and culture conditions. Strains and plasmids are detailed in the supplemental material. *Escherichia coli* strains were grown in Luria-Bertani broth supplemented with 100 μg of ampicillin ml^{-1}, 500 μg of hygromycin (Hm) ml^{-1}, 50 μg of kanamycin (Km) ml^{-1}, or 25 μg of chloramphenicol ml^{-1}. Standard methods were used for *E. coli* transformation, DNA manipulation (24), and the preparation of *Streptomyces lividans* TK24 protoplasts and *S. lividans* transformation (16). *S. lividans* transformants were selected after the addition of 5 mg of Hm or Km to 3 ml of soft agar overlay and purified on yeast extract or malt extract agar (16) with 250 μg of Hm or Km ml^{-1}.

MIC of 5FC or 5FU. 5FC was prepared as a stock solution of 15 mg ml^{-1} in distilled water, and the solution was sterilized by filtration. 5FU was prepared as a stock solution of 5 mg ml^{-1} in dimethyl sulfoxide. MICs were determined by the agar dilution method (3) as detailed in the supplemental material.

Construction of genes for selection. A cytosine deaminase gene optimized for expression in actinomycetes, *codA*(*s*), was developed from the *E. coli* CodA sequence (5) by reverse translation and codon optimization (9). An SphI site at the initiation codon (CGATGC, where underlining indicates the codon) was created, resulting in a Ser→Pro mutation of the second residue of CodA. The coding segment was synthesized by Geneart AG (Regensburg, Germany). The promoter from pFDNeo-S (6) was inserted upstream from the coding sequence. The *codA*(*s*) sequence has been deposited at NCBI (accession number EU099038). A D314A mutant version (19) named *codA*(*sm*) was also created by PCR-directed mutagenesis (13).

For positive selection, we used the aminoglycoside resistance gene from pFDNeo-S, providing this gene with the promoter from the *aacC4* gene (1), to obtain *neoA*. *S. lividans* TK24 harboring a single copy of *neoA* (strain TK24 *neoA*) was resistant to 600 μg ml^{-1} of Km (data not shown).

Vector construction. The *codA*(*s*) cassette was inserted into Litmus 38i (7). We then added *neoA* and deleted the Ap^r gene and the origin of replication of M13. Finally, we introduced

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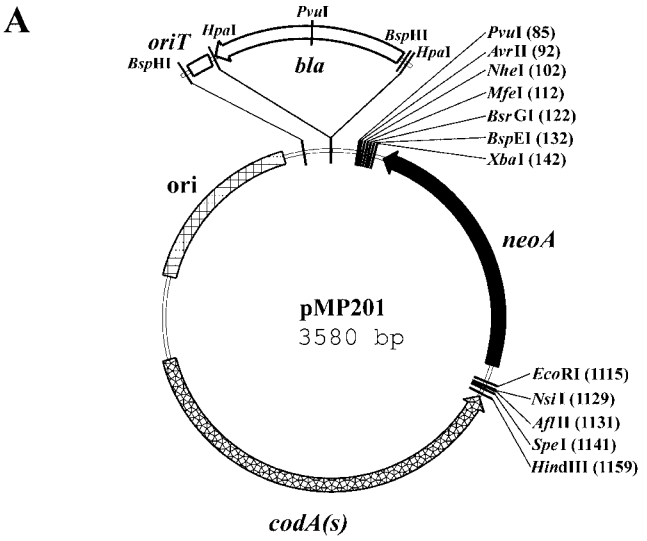

B

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81          91          101         111         121         131         141
-           -           -           -           -           -           -
PvuI        AvrII       NheI        MfeI        BsrGI       BspEI       XbaI
cg at/cggactc/ctag gatgcg/ctag ctcagc/aatt gcagtt/gtac atagct/ccgg actgct/ctag a
gc/ta gcctgag gatc/ctacgc gatc/gagtcg ttaa/cgtcaa catg/tatcga ggcc/tgacga gatc/t

          1121        1131        1141        1151        1161
          -           -           -           -           -
EcoRI       NsiI    AflII       SpeI                HindIII
g/aatt cgtcaa tgca/tc/ttaa gatcga/ctag tgactcctgcagga/agct t
c ttaa/gcagtt/acgt ag aatt/ctagct gatc/actgaggacgtcct tcga/a
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FIG. 1. Vector pMP201 and derivatives. (A) Map of the pMP201 vector with polylinkers XbaI-PvuI and HindIII-EcoRI. *neoA*, aminoglycoside resistance gene with the promoter from the *aacC4* gene; *codA*(*s*), synthetic version of the cytosine deaminase gene; ori, origin of replication for *E. coli*. The positions of oriT (origin of conjugative transfer), present in pMP301 and pMP302, and *bla* (ampicillin resistance gene), present in pMP302, are also shown. Note that the PvuI site in the polylinker is not unique in derivatives containing the *bla* gene. (B) DNA sequences of polylinkers in pMP201 and derivatives (coordinates in both panels are from the pMP201 sequence, and those in panel B correspond to the first boldface base pair for each restriction enzyme). Boldface letters and dashes indicate the restriction sites and the respective cleavage sites.

two newly designed polylinkers, obtaining the vector pMP201 (Fig. 1). Polylinker HindIII-EcoRI was introduced between *codA*(*s*) and *neoA*, while polylinker XbaI-PvuI was inserted downstream from *neoA* (Fig. 1). Both polylinkers will accommodate DNA fragments in insertional gene disruption procedures, while only the polylinker XbaI-PvuI will be used for cloning the fragments involved in gene replacement, including in-frame deletion fragments. Replacing *codA*(*s*) by the mutated version *codA*(*sm*), we obtained pMG201M (see Fig. S1 in the supplemental material).

The conjugative vector pMP301 [Km^r *codA*(*s*) oriT] was obtained by inserting the transfer origin oriT from pIJ773 (10) into pMP201. To allow the use of our method with Km^r *E. coli* donor strains, an Ap^r derivative, pMP302 [Ap^r Km^r *codA*(*s*) oriT], was constructed. Construction is detailed in the supplemental material.

Resistance to 5FC is frequent among actinobacteria. The growth of CodA-positive *E. coli* is inhibited at 20 μg ml^{-1} of 5FC in minimal medium (21). In contrast, concentrations as high as 400 to 800 μg ml^{-1} were necessary to inhibit the growth of tested actinobacteria (Table 1). Most strains were very sensitive to 5FU. The absence of cytosine deaminase, observed in

TABLE 1. MICs

Species and/or strain	MIC (μg ml^{-1}) of:	
	5-FC	5-FU
Amycolatopsis orientalis subsp. *orientalis* ATCC 19795	800	5
Mycobacterium smegmatis MC^{2}155	>800	20
"*Nocardia lactamdurans*" NRRL 3802	800	10
Nocardioides sp. strain N106	>800	0.4
Saccharopolyspora erythraea ATCC 11635	400	<0.1
Streptomyces antibioticus Tü 1718 (SKB)	>800	40
Streptomyces avermitilis ATCC 31267	>800	>40
Streptomyces avidinii ATCC 27419	>800	40
Streptomyces clavuligerus NRRL 3585	800	<0.1
S. coelicolor A3(2) M145	800	<0.1
Streptomyces fradiae	400	<0.1
S. griseus B2682	800	<0.1
S. lividans TK24	800	<0.1
Streptomyces melanosporofaciens EF-76	>800	<0.1
Streptomyces scabiei EF-35	>800	0.8
Streptomyces venezuelae ATCC 15439	800	<0.1
S. lividans TK24 mutant strains		
TK24 *neoA*	800	≤0.1
TK24 *codA*(*s*)	200	≤0.1
TK24 *codA*(*sm*)	20	≤0.1
TK24 Δ*csnA*::*neoA*	800	≤0.1
TK24 Δ2657h	800	≤0.1

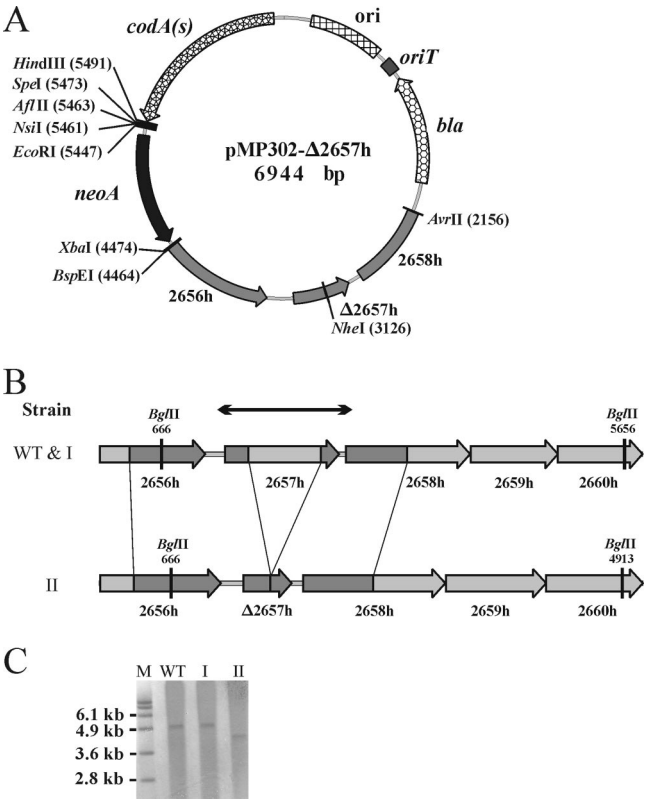

FIG. 2. Inactivation of an *S. lividans* SCO2657 homolog gene (2657h) via replacement by an in-frame deletion. (A) Map of the pMP302 vector derivative (pMP302-Δ2657h) harboring PCR-amplified *S. lividans* genome fragments for homologous recombination. Positions of restriction sites are listed in parentheses. (B) Diagrams showing the wild-type (WT) genomic section of *S. lividans* and its configuration after the second crossing over. I, strain with a reversion to the wild-type configuration after two recombination events involving the same PCR-amplified fragment; II, strain with an in-frame deletion obtained by recombination events occurring alternatively in both amplified segments; 2656h to 2660h, *S. lividans* homologs of the respective *S. coelicolor* A3(2) genes; arrows, DNA fragment used as a probe in Southern blotting. Positions of restriction sites are given. (C) Southern blotting analysis of the 2657h gene area before (WT) and after (I or II) selection steps. Genomic DNA from the host strain (WT) and two PCR-preselected clones (I and II) resistant to 5FC was digested with BglII and analyzed by Southern blotting. M, molecular size markers.

S. coelicolor and *Streptomyces griseus* (15), seems to be frequent and is perhaps generalized among actinobacteria. The CodA-based counterselection system could then be applicable to a vast array of species.

Cytosine deaminase increases 5FC sensitivity in *S. lividans*. We synthesized de novo the entire coding sequence for CodA, improving its codon adaptation index (25) for *S. lividans* from 0.271 to 0.991. *S. lividans* TK24 harboring a single *codA*(*s*) gene [strain TK24 *codA*(*s*)] was four times more sensitive to 5FC than its parent strain (Table 1). Its growth and sporulation phenotypes remained unchanged.

The D314A mutant form of CodA has 17-fold lower activity against native cytosine, while its activity against 5FC is 2-fold higher (19). *S. lividans* TK24 harboring one copy of the mutated *codA*(*sm*) gene [strain TK24 *codA*(*sm*)] was 10 times more sensitive to 5FC than TK24 *codA*(*s*) and 40 times more sensitive than the native TK24 (Table 1), allowing counterselection at lower 5FC concentrations.

***codA*(*s*)-based counterselection.** We first introduced an in-frame deletion into the *S. lividans* homolog of the SCO2657 gene (designated 2657h) encoding a putative ROK family protein (27). Disruption cassettes amplified from the *S. lividans* genome were cloned into pMP302, and *E. coli* ET12567 (pUZ8002) was then transformed with the resulting plasmid. After conjugation with *S. lividans* spores, one single-crossover Km^r clone for each cell/spore ratio (1:1 and 40:1) was obtained, resulting in a recombination frequency of 5×10^{-7}. In the counterselection step, the plating of 20,000 spores onto MM with 200 μg of 5FC ml^{-1} yielded 18 resistant colonies, resulting in a second-crossover frequency of $\sim 10^{-3}$. Figure 2B summarizes the recombination events. Of 12 purified clones, 11 regained the original gene configuration while 1 harbored the in-frame deletion, as shown by Southern blotting (Fig. 2C).

We used pMG201M for the disruption of the *S. lividans* TK24 chitosanase gene (*csnA*), an ortholog of SCO0677 from *S. coelicolor* A3(2) encoding an endochitosanase (17). *S. lividans* TK24 was transformed with the plasmid pMG201M-Ω*csnA* (see the supplemental material). A Km^r colony was picked up, and its spores were plated onto MM with Km (250 μg ml^{-1}) and 5FC (50 μg ml^{-1}). 5FC-resistant colonies appeared at a 7×10^{-3} frequency. Fragments of the expected lengths were observed by on-colony PCR performed with two 5FC-resistant colonies, indicating the successful disruption of the *csnA* gene (data not shown).

Conclusion. The cytosine deaminase gene can be efficiently used as a negative selection marker for gene disruption and replacement in *Streptomyces*. The method can be used for any actinobacterial species naturally resistant to 5FC while sensitive to 5FU. It should be a valuable addition to the actinobacterial genetic toolbox.

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