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The *aadA* Gene of Plasmid R100 Confers Resistance to Spectinomycin and Streptomycin in *Myxococcus xanthus*

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Plasmids with the *aadA* gene from plasmid R100, which confers resistance to the aminoglycosides spectinomycin and streptomycin in *Escherchia coli*, can be introduced into wild-type *Myxococcus xanthus*, strain DK1622, by electroporation. Recombinant *M. xanthus* strains with integrated plasmids carrying the *aadA* gene acquire resistance to high levels of these antibiotics. Selection for *aadA* in *M. xanthus* can be carried out independently of, or simultaneously with, selection for resistance to kanamycin. The kinds and frequencies of recombination events observed between integrative plasmids with *aadA* and the *M. xanthus* chromosome are similar to those observed after the transformation of yeast. Cleavage of integrative plasmid DNA at a site adjacent to a region of homology between the plasmid and the *M. xanthus* genome favors the targeted disruption of *M. xanthus* genes by allele replacement.

The soil bacterium *Myxococcus xanthus* is a model prokaryotic system for the study of intercellular communication. Cell-cell interactions play central roles in both gliding motility and an elaborate multicellular developmental cycle initiated by starvation. Development leads to the morphogenesis of fruiting bodies containing differentiated, heat-resistant spores. Although the change from rod-shaped vegetative cells to spherical spores is rapid, genetic methods have been used successfully to arrange the early steps of this process into an ordered series of molecular events. Many mutations that block *M. xanthus* development do not affect vegetative growth. Consequently, methods for the analysis of mutations that block development have relied on genetic manipulations with vegetative cells.

Following the discovery that transposon Tn*5* is active in *M. xanthus* (14), Tn*5-lac*, among the first hybrid transposons used to generate reporter gene fusions, was constructed and used in *M. xanthus* (11). The isolation of transposon-generated fusions of *lacZ* with developmentally induced promoters has resulted in a set of temporal signposts that distinguish different developmental stages (12, 13). In addition, *M. xanthus* has generalized transducing phages (5, 18). which permit fine-structure mapping. One of these phages, Mx8, is temperate and integrates as a prophage at a preferred chromosomal locus (*attB*). Because plasmid origins of replication that function in other gram-negative bacteria do not function as origins in *M. xanthus*, these plasmids can be used as integrative vectors if they carry either a region of homology with the *M. xanthus* genome (19, 24, 26) or the Mx8 *int-attP* region (15, 20).

Because *M. xanthus* has a high frequency of homologous recombination, the integration of plasmids by homologous recombination can be used to generate merodiploids and exchange engineered alleles with chromosomal alleles of targeted genes (19). Thus, plasmids with cloned *M. xanthus* inserts can be used in the same way that yeast integrative plasmids (yIPs) are used to manipulate the *Saccharomyces cerevisiae* genome (8, 21). Alternatively, plasmids can be integrated at the *attB* locus to construct more-stable merodiploids for complementation tests (24, 26). Plasmids can be introduced into *M. xanthus* efficiently by using the improved method for electroporation we developed (10).

The rules for homologous recombination between a plasmid and the *M. xanthus* genome appear to be similar to those for recombination between yIPs and the *S. cerevisiae* genome (21). Both circular and linear plasmid DNAs give rise to recombinants, and electroporation with linear plasmid DNA often results in the integration of a circular form of the plasmid, presumably mediated by gap repair of double-stranded ends. Gap repair of the ends of linear plasmids poses a significant problem for the construction of gene disruptions, because single crossover events between a repaired plasmid and the genome, leading to cointegrate formation, can be more frequent than double crossover events leading to allele replacement.

To date, only the kanamycin resistance (Km^r) determinants derived from Tn*5* (14) or Tn*903* (25) have worked well in both *Escherichia coli* and *M. xanthus* hosts. Unfortunately, *M. xanthus* is naturally resistant to gentamicin. Although tetracycline resistance determinants have been shown to function in *M. xanthus* (2), these determinants work poorly when it is grown in its preferred, rich medium supplemented with Mg^{2+}. Growth in the presence of Mg^{2+} likely facilitates a natural mechanism for tetracycline export and thereby leads to a high background of tetracycline-resistance phenocopies. Although resistance to tetracycline mediated by the Tn*10 tetA* determinant works well in *M. xanthus*, selection for this gene on high-copy-number plasmids in *E. coli* is lethal (4), unless the cells are plated on media with oxytetracycline. The *tetA* determinant of plasmid pBR322, which works well in high copy numbers in *E. coli*, works relatively poorly in *M. xanthus* (our unpublished observations). Li and Shimkets have used the trimethoprim resistance determinant for plasmid R338 successfully in *M. xanthus* (16). We have found that its success in *M. xanthus* depends on the relative concentration of competing folates in rich, Casitone-containing medium and that selection for this marker in many *E. coli* hosts can be problematic.

As part of our search for additional antibiotic resistance determinants that function well in both *M. xanthus* and *E. coli*,

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TABLE 1. Efficiencies of electroporation of *int-attP* plasmids into wild-type *M. xanthus* (strain DK1622).

Plasmid	Reference	Size (kb)	Marker(s)[a]	EOE (μg^{-1})[b]
pBGS18	25	3.6	Km^r	0
pAY721	23	5.8	Km^r *int-attP* region	2.6 ± 10^4
pGB2	6	4.0	Sp^r Sm^r	1.6 ± 10^2
pAY952	17	6.2	Sp^r Sm^r *int-attP* region	3.6 ± 10^4

[a] Plasmids carry either the *npt!* gene (Km^r determinant) from transposon Tn*903* or the *aadA* gene (Sp^r Sm^r determinant) from conjugative plasmid R100; a subset of plasmids also encode the *int-attP* site-specific recombination functions from temperate *M. xanthus* phage Mx8.

[b] Efficiencies of electroporation (EOE) were determined from the average results of at least four independent experiments, in which 100 to 200 ng of DNA was used to electroporate 10^9 wild-type cells as described previously (10). The value of 1.6 ± 10^2electroporants/μg for pGB2 DNA represents the background of spontaneous Sp^r Sm^r mutants; all of these (23 of 23 tested) did not repurify as resistant colonies. Spontaneous resistant mutants can be also distinguished from recombinants carrying integrated plasmids by amplifying purified *M. xanthus* DNA from resistant strains with the primers TM5 (CCCCAAGCTTGGTACC ACTAGTTATTTGCCGACTACCTTGGTGA) and TM6 (AAAAAAGCTTCC ATGGTTTCATGGCTTGTTATGACTG), specific for the *aadA* gene and its promoter, respectively. In addition, these primers permit amplification of the *aadA* gene and its promoter from plasmid templates for direct cloning into sites within *M. xanthus* genes.

we tested whether the *aadA* gene of plasmid R100 might confer resistance to both spectinomycin (Sp^r) and streptomycin (Sm^r) in *M. xanthus*. Campos and coworkers have shown that *M. xanthus* is sensitive to both antibiotics (5). Consistent with this, we found that wild-type *M. xanthus* (strain DK1622) (9) plated with low efficiencies (8×10^{-7}) on rich CTPM (28) medium supplemented with either 0.8 mg of spectinomycin per ml or 1.0 mg of streptomycin per ml. On medium with both antibiotics, *M. xanthus* plated with an even lower efficiency (4×10^{-7}), and usually >90% of the colonies formed after 5 days at 32°C did not grow when repurified on the same medium. Presumably, the potency of these antibiotics decreased during the long (5- to 7-day) incubation times required for the growth of *M. xanthus* colonies. Consistent with this idea, the frequency of resistant phenocopies is greater on plates that have been stored at room temperature for several days before use.

To test whether the *aadA* gene confers antibiotic resistance on *M. xanthus*, we constructed plasmid pAY952 (17). This plasmid was constructed by adding the 2.2-kb fragment of Mx8 DNA with the *int* and *attP* genes to plasmid pGB2 (6), which has both the *aadA* gene and an origin of replication derived from R100. Upon electroporation of pAY952 into DK1622, Sp^r Sm^r recombinants arose at a frequency of about 200-fold above the backgroud frequency of spontaneous resistant mutants (Table 1), indicating that the *aadA* gene functions in *M. xanthus*.

To demonstrate that this Sp^r Sm^r marker can be used in combination with selection for kanamycin resistance, we constructed a cointegrate plasmid, pAY1074, with an insertion of pGB2 in the *fibR* gene (28). The *fibR* gene is located within a 3.5-kb region of the *M. xanthus* genome which we subcloned from plasmid pAY694 (28) into plasmid vector pBGS18, which has the Km^r determinant of transposon Tn*903* (25), to make

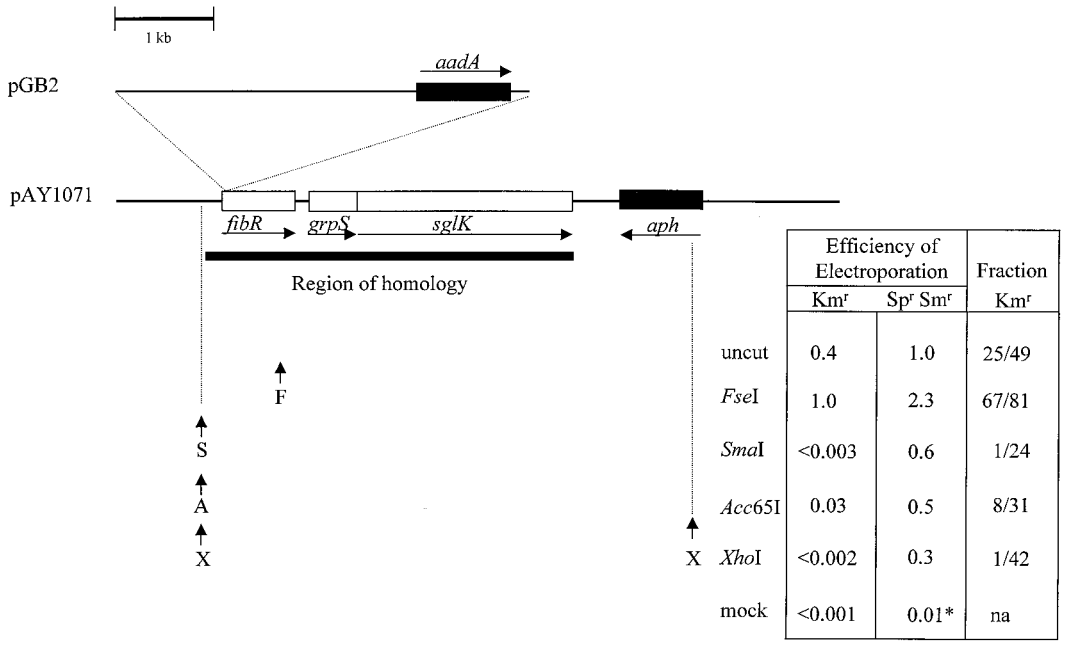

FIG. 1. Cleavage of plasmid DNA prior to electroporation into *M. xanthus* affects the frequency and nature of recombination events between the plasmid and chromosome. Shown to approximate scale in the upper left is the structure of plasmid pAY1074, which is Km^r (*aph*) plasmid pAY1071 with an insertion of Sp^r Sm^r (*aadA*) plasmid pGB2 in the *M. xanthus fibR* gene. The region of homology carried by the plasmid is 3.5 kb in length (28). The efficiencies of electroporation of closed circular plasmid pAY1074 DNA (uncut) and pAY1074 DNA cleaved at the indicated sites are compared on the right. The first two columns are numbers representing the independent measurements of Km^r and Sp^r Sm^r recombinants arising from the same electroporations and are the averages of six independent determinations. Fractions shown in the third column were obtained by testing individual Sp^r Sm^r electroporants obtained from two independent determinations for a Km^r phenotype. All efficiencies of electroporation are given realtive to the efficiency of Sp^r Sm^r recombinants arising with uncut pAY1074 DNA ($4.5 \times 10^3 \pm 2.7 \times 10^3$ μg^{-1}). Cleavage of pAY1074 DNA at its unique *Fse*I site within the region of homology stimulated the formation of recombinants, whereas cleavage at sites at the junction of, or past, the region of homology reduced the frequency of Sp^r Sm^r recombinants. Prior cleavage at sites outside the region of homology reduced the frequency of recovery of Km^r Sp^r Sm^r conintegrates between the plasmid and chromosome. Km^s Sp^r Sm^r recombinants were favored, presumably after double-recombination events flanking the pGB2 insertion resulted in the desired allele replacement event. The asterisk indicates that more than 90% of the colonies obtained on medium with both antibiotics, spectinomycin and streptomycin, after mock electroporation (with no plasmid DNA) did not grow when repurified on the same medium, na, not applicable.

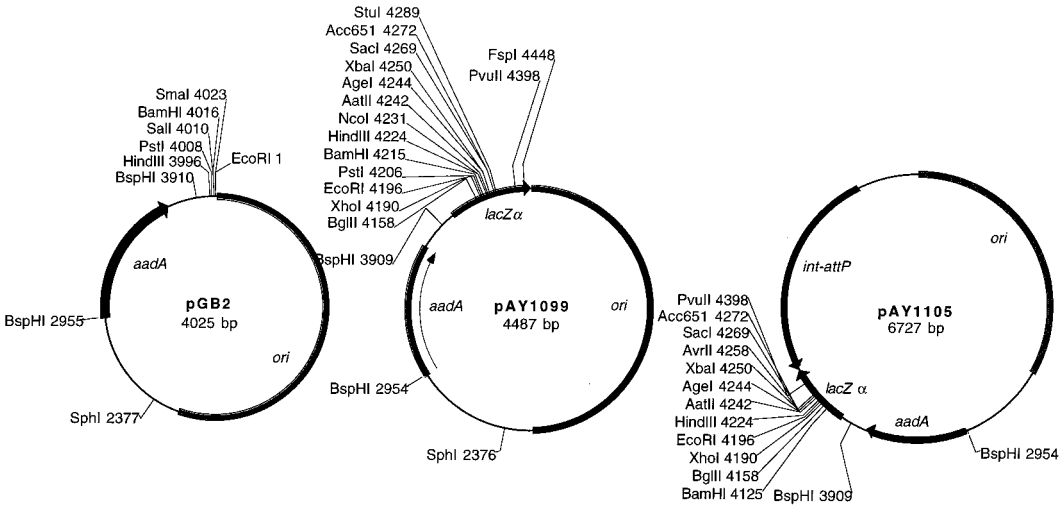

FIG. 2. Structures of plasmids pGB2, pAY1099, and pAY1105. The *aadA* gene is flanked by a pair of *Bsp*HI sites in each vector, and its translation initiates at an ATG codon within one of these sites. Also shown are unique sites within the polylinker of pGB2 derived from pUC8 and within the fragment of the *lacZ* gene carried by plasmids pAY1099 and pAY1105 derived from pLITMUS28 (New England Biolabs). The positions of the *aadA* gene from plasmid R100, the region from R100 including its origin of replication (*ori*) (also present in *Salmonella* plasmid pSC101), the region of the *E. coli lac* operon encoding the β-complementing fragment of LacZ, and the *int* and *attP* genes from myxophage Mx8 are indicated. Text files of the sequences of these plasmids and detailed notes on their construction are available by e-mail upon request.

pAY1071. To build pAY1071, the 3.5-kb *Bgl*II-*Hin*dIII fragment of pAY694 was ligated to the *Bam*HI and *Hin*dIII sites of pBGS18. To build pAY1074, pAY1071 was cleaved at a unique *Age*I site within *fibR*, pGB2 was cleaved at its unique *Xma*I site in its polylinker, and the plasmids were ligated together.

We electroporated both circular plasmid DNA and plasmid DNA treated with several different restriction endonucleases into DK1622, selected for Km^r Sp^r Sm^r or Sp^r Sm^r recombinants, and screened the Sp^r Sm^r recombinants for the Km^r phenotype. Four conclusions can be drawn from the results shown in Fig. 1. First, when a closed circular plasmid carrying a Sp^r Sm^r insertion in a 3.5-kb region of homology with the *M. xanthus* genome is electroporated into *M. xanthus*, about half of the recombinants are Km^r cointegrates, and the other half are Km^s strains in which an allele replacement event has occurred. Second, cleavage of the plasmid at a unique site within the region of homology (*Fse*I) stimulates recombination between the plasmid and chromosome. This result suggests that, as in yeast (21), DNA ends are recombinogenic in *M. xanthus*. However, cleavage at a unique site within the region of homology (such as the *Fse*I site of pAY1074) does not discourage the formation of cointegrates. Third, cleavage of plasmid pAY1074 at a unique site past the end of the region of homology (*Sma*I or *Acc*65I) does not stimulate the yield of recombinants, as it does for some yIPs (21). However, it favors the recovery of Sp^r Sm^r strains resulting from allele replacement events, and this bias is more pronounced with *Sma*I, which generates a blunt-ended linear module. Fourth, cleavages of the plasmid at two sites (with *Xho*I), one at the junction of the homologous region and a second with the Km^r determinant of the plasmid vector backbone, have similar effects on the frequency and types of recombinants. In both *M. xanthus* and *S. cerevisiae*, illegitimate recombination events between homologous substrates and the genome are almost never observed, and the similarities between the recombinational fates of circular and linear plasmid DNAs in these hosts are striking. Thus, the simplest interpretation of our results is that, as in *S. cerevisiae*, double-stranded DNA ends are the preferred substrates for the *M. xanthus* recombinational machinery.

These results show that a simple procedure can be used to cross an insertion marked by an antibiotic resistance determinant onto the *M. xanthus* chromosome. A plasmid carrying the insertion should be cleaved at one or both ends of its region of homology with the chromosome prior to electroporation to discourage the formation of cointegrates. We have obtained similar results after cleavage and electroporation of plasmids with several different subcloned regions of the *M. xanthus* genome. In each case, cleavage of a plasmid at one or both ends of the region of homology with the chromosome favors the recovery of recombinants in which allele replacement events have occurred. This method for generating allele replacements on the *M. xanthus* genome is simpler than the previous methods, which have relied on two sequential selections for the integration and excision of plasmids carrying the selectable Km^r determinant and the counterselectable *E. coli galK* (27) or *Bacillus subtilis sacBR* (29) genes.

We note that the electroporation of linear DNA molecules derived from circular plasmids into several other bacteria has been used successfully to generate allele replacements. Linearization of a plasmid carrying a subcloned *Haemophilus ducreyi* gene interrupted by an antibiotic resistance determinant also appears to stimulate allele exchange (7). The more recent demonstrations that linear DNA molecules permit allelic exchange in *Mycobacterium bovis* BCG (1), *Mycobacterium tuberculosis* (3), and *Borrelia burgdorferi* (22) will facilitate the genetic analysis of these pathogens in the near future.

To increase the versatility of Sp^r Sm^r plasmids as shuttle vectors for use in both *E. coli* and *M. xanthus*, we constructed a derivative of pGB2, pAY1099, with the α-complementing fragment of the *lacZ* gene from plasmid pLTMUS28 (New England Biolabs). Primers 5′ GGAGGGTGGCCAAATGTG AGTTAGCTCACTCA and GCCGGCCAATTGTTATTAC CAAGCGAAGCGCC were used to amplify bp 2315 to 2798 of pLITMUS28, and the amplified product was cleaved with *Msc*I and *Mfe*I and ligated to the *Eco*RI and filled-in *Hin*dIII sites of pGB2 (6). This amplified fragment has many unique cloning sites within a polylinker located at the 5′ end of the *lacZ* gene. Many recombinant plasmids with inserts in this

polylinker can be screened by α-complementation in an appropriate *E. coli* host, such as JM107 (30), in the presence of chromogenic substrates for β-galactosidase.

We also made a derivative of pGB2 with both the α-complementing fragment and the Mx8 *int-attP* region present in pAY952. pAY952 was cleaved with *Eco*RI and *Acc*65I, ends were filled in, and the plasmid backbone was ligated to make plasmid pAY1103. The amplified fragment of pLITMUS28 was cleaved with *Msc*I and *Mfe*I and ligated to the filled-in *Hin*dIII site of pAY1103 to make pAY1105. When derivatives of pAY1105 are electroporated into *M. xanthus*, they prefer to integrate at the *attB* bacterial attachment site for prophage Mx8, even if they carry a region of homology with the *M. xanthus* genome (data not shown). The structures of these integrative shuttle vectors, which may have more general uses in other myxobacteria or gram-negative hosts sensitive to the aminoglycosides spectinomycin and streptomycin, are shown in Fig. 2.

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