APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1991, p. 3522–3528
0099-2240/91/123522-07$02.00/0

Vol. 57, No. 12

Stability of the δ-Endotoxin Gene from *Bacillus thuringiensis* subsp. *kurstaki* in a Recombinant Strain of *Clavibacter xyli* subsp. *cynodontis*

JOHN T. TURNER,[1] JAY S. LAMPEL,[2]* ROBERT S. STEARMAN,[2] GEORGE W. SUNDIN,[1]†
PAUL GUNYUZLU,[2]‡ AND JAMES J. ANDERSON[2]§

Microbial Ecology Group[1] and Molecular Genetics Group,[2] Crop Genetics International, 7170 Standard Drive, Hanover, Maryland 21076

Received 15 May 1991/Accepted 18 September 1991

Deletion of chromosomally inserted gene sequences from *Clavibacter xyli* subsp. *cynodontis*, a xylem-inhabiting endophyte, was studied in vitro and in planta. We found that nonreplicating plasmid pCG610, which conferred resistance to kanamycin and tetracycline and contained segments of *C. xyli* subsp. *cynodontis* genomic DNA, integrated into a homologous sequence in the bacterial chromosome. In addition, pCG610 contains two copies of the gene encoding the CryIA(c) insecticidal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD73. Using drug resistance phenotypes and specific DNA probes, we found that the loss of all three genes arose both in vitro under nonselective conditions and in planta. The resulting segregants are probably formed by recombination between the repeated DNA sequences flanking pCG610 that resulted from the integration event into the chromosome. Eventually, segregants predominated in the bacterial population. The loss of the integrated plasmid from *C. xyli* subsp. *cynodontis* revealed a possible approach for decreasing the environmental consequences of recombinant bacteria for agricultural use.

The use of recombinant DNA techniques to produce microbial products for agriculture has focused attention on risk assessment of genetically engineered microorganisms. Among the many important components of risk assessment are dispersal, persistence, biological activity, and genetic stability. Some recombinant microbes may be unable to persist for long periods of time because of environmental conditions at the delivery site. Alternatively, if a genetically altered microbe survives the physical, chemical, and biological stresses present at the delivery site or is dispersed to a more favorable niche, then the biological activity and genetic stability of the organism become key factors in assessing the environmental risk associated with the release.

Loss of introduced genes resulting from deletions of DNA sequences in plasmids or loss of entire plasmids has been reported in recombinant strains of *Bacillus subtilis* (4, 11, 14), *Bacillus stearothermophilus* (2), *Escherichia coli* (3), and *Streptococcus pneumoniae* (4, 5). Many cases of deletions appear to be related to homologous sequences that flank the deleted segments (3, 5, 11, 13). Albertini et al. (3) showed that the incidence of deletions that are 700 to 1,000 bp long decreased as the level of homology of the flanking repeated sequences decreased.

Methods for monitoring the fate of environmentally released bacteria have been developed. Among these methods are the *lacZY* system for fluorescent pseudomonads (10), the enzymatic activity of a deregulated 2,4-dichlorophenoxyacetate monooxygenase (16), and a *Vibrio* bioluminescence gene set to track phytopathogenic bacteria (21). In a microcosm release of a pseudomonad population, recombinant plasmids encoding a *xylE* marker gene were used for bacterial detection (20); the workers observed differential segregation rates of *xylE* DNA that appeared to be dependent on bacterial growth rates.

Recently, integrative transformation systems have been described for *Candida tropicalis* (12), *Bacillus alcalophilus* (22), and *Pseudomonas cepacia* (13). In the case of soil-inhabiting *P. cepacia*, a specific deletion of the chromosomally inserted DNA sequences occurred between repeated flanking sequences, and the frequency of sequence loss appeared to be related to environmental stress (13). Workers have also described deletions between sites that are not contained within directly repeated sequences, possibly because of the action of a topoisomerase (18).

Our work has focused on genetically altering an endophytic bacterium, *Clavibacter xyli* subsp. *cynodontis*, so that it contains a nonreplicating plasmid inserted into the chromosome. The integrated plasmid contains the δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* HD73 fused to a gene that confers kanamycin resistance, a tetracycline resistance gene, and an *E. coli* replicon. The wild-type strain of *C. xyli* subsp. *cynodontis* described by Davis et al. (9) is found frequently in nature colonizing the vascular system of Bermuda grass (*Cynodon dactylon* L.) (8, 17). In addition to Bermuda grass, *C. xyli* subsp. *cynodontis* and engineered strains of *C. xyli* subsp. *cynodontis* containing the δ-endotoxin gene have been shown to colonize several important crop plants (16a), including corn (*Zea mays* L.). High populations of *C. xyli* subsp. *cynodontis* are distributed systemically in the xylem elements of corn following inoculation (20a), and for this reason *C. xyli* subsp. *cynodontis* is being developed as a delivery system for biopesticides. *C. xyli* subsp. *cynodontis* MDR1.3 is a prototype version that was tested in corn for control of the European corn borer (*Ostrinia nubilalis*) in greenhouse and field experiments via expression of the δ-endotoxin gene. The genetic stability of

* Corresponding author.
† Present address: Department of Plant Pathology, Oklahoma State University, Stillwater, OK 74078.
‡ Present address: Department of Molecular Biology, Sterling Drug Company, Langhorn, PA 19355.
§ Present address: National Institutes of Health, General Medical Sciences, Bethesda, MD 20892.

such a construction could potentially have an impact on both the efficacy of the biopesticide and environmental risk. In this study we examined both the deletion of inserted gene sequences and the ecological ramifications of such deletions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *C. xyli* subsp. *cynodontis* MDE1 was originally isolated from Bermuda grass growing in eastern Maryland. *C. xyli* subsp. *cynodontis* was routinely cultured on SCM medium (9) or on GC medium. GC medium contained (per liter) 4.8 g of Bacto Soytone, 3.2 g of Bacto Tryptone, 0.35 g of K_2HPO_4, 1.1 g of KH_2PO_4, 2 g of $MgSO_4 \cdot 7H_2O$, and 8 g of Gelrite (Microbiological Sciences, Inc., Fiskeville, R.I.). After autoclaving, 50 ml of a filter-sterilized solution containing 2 g of glucose and 0.5 g of cysteine was added. The liquid medium which we used was designated S-27 medium; this medium contained the same ingredients as GC medium except that 1 g of $MgSO_4 \cdot 7H_2O$ was added and Gelrite was omitted. The concentrations of antibiotics which we used were as follows: kanamycin, 200 μg/ml; tetracycline, 2 μg/ml.

E. coli DB4729, which was a *recA* derivative of strain MM294 and was provided by D. Botstein, was used in all of the cloning steps to make integration plasmid pCG610, which contained the *B. thuringiensis* subsp. *kurstaki* HD73 δ-endotoxin gene. Cells were grown at 37°C in Luria-Bertani broth (19). Transformants with antibiotic resistance were grown in the presence of ampicillin (final concentration, 100 μg/ml).

DNA manipulation. High-molecular-weight genomic DNA was isolated from *C. xyli* subsp. *cynodontis* by gently lysing cells embedded in agarose plugs. Cells were grown to mid-log phase in S-27 medium supplemented with glycine (final concentration, 0.5%). A 1.5-ml aliquot of harvested cells was washed twice with ET buffer (50 mM EDTA, 10 mM Tris-HCl; pH 7.8). The final cell pellet was resuspended in 150 μl of ET buffer and warmed to 40°C. A lysozyme solution (10 μl of a 20-mg/ml stock solution made in ET buffer) was added to each tube, and then 0.25 ml of 1.0% SeaPlaque Agarose (FMC BioProducts, Rockland, Maine) (made in 0.125 M EDTA–2.5 mM Tris-HCl, pH 7.8) was added. The tubes were then quick-chilled on ice. The cells were prepared for lysis by adding 0.4 ml of LET buffer (0.5 M EDTA, 10 mM Tris-HCl; pH 7.8) and then incubating the preparation overnight at 37°C. The liquid phase was removed from the tubes containing the agarose plugs, and 0.4-ml aliquots of a proteinase K solution (1-mg/ml stock solution made in LET buffer supplemented with 0.5% sodium dodecyl sulfate) were added to complete the lysis step. The tubes were incubated overnight at 50°C. The liquid phase was again removed, and the agarose plugs containing the DNA were stored at 4°C and melted at 70°C immediately before use.

To characterize phenotypic segregants, DNA probes were prepared from plasmids containing tetracycline and kanamycin resistance genes and the δ-endotoxin gene. The probes were purified by preparative agarose gel electrophoresis, which was followed by excision of the appropriate band and final recovery of the DNA by electroelution. The probes were labeled by using a Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions.

DNA probes for Southern blot analysis of *C. xyli* subsp. *cynodontis* transformants were labeled with [α-^{32}P]dCTP (NEN Research Products, Boston, Mass.) by using the Nick Translation System (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The DNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, N.H.) as described by Maniatis et al. (19). For the Southern blot analysis the prehybridization and hybridization solutions contained 1 M NaCl, 5× Denhardt solution, 0.2% sodium dodecyl sulfate, 2 mM EDTA, 20 μg of sonicated calf thymus DNA per ml, and 50 mM Tris-HCl (pH 8.0). The methods used for hybridization of the labeled DNA to the Nytran membranes and development of the radioactive blots have been described previously (19).

Filter preparation for dot blot segregation analysis. Cell suspensions were prepared from isolated colonies having known antibiotic resistance phenotypes. Approximately 10^8 cells per well were loaded onto nitrocellulose filters (Schleicher & Schuell) by using a 96-well vacuum manifold (American Bionetics, Hayward, Calif.). The cells were lysed by placing the filters, cell side up, on Whatman 3MM blotting paper that was saturated with 0.5 N NaOH–1% sodium dodecyl sulfate for 15 min. The excess cell debris was removed from the filters by using a surgical scrub sponge (American Cyanamid Co., Danbury, Conn.) that was saturated with 0.5 N NaOH–1% sodium dodecyl sulfate. The filters were rinsed briefly in sterile water and neutralized by soaking them in 1.0 M Tris-HCl (pH 8.0) for 15 min. The filters were then baked for 2 h at 80°C prior to hybridization. The hybridization conditions and buffers used for the segregation dot blot analysis in which we used the Genius nonradioactive DNA labeling and detection kit were the conditions and buffers described by the manufacturer.

In planta segregation studies. Bermuda grass plants that were preassayed and were found to be free of naturally occurring *C. xyli* were grown in a glasshouse in pots containing approximately 4 liters of potting mixture. The plants were maintained by watering them daily and fertilizing them twice a week with Nutriculture 20-20-20 plus micronutrients (Plant Marvel Laboratories, Chicago Heights, Ill.). The plants were trimmed every 1 to 2 weeks to contain them within the pots. Once transplants were established, their stems were inoculated by using an inoculating device which was constructed from a sewing needle (23) and delivered 0.75 μl of a preparation of strain MDR1.3 (which was strain MDE1 containing pCG610 as a chromosomal integration) having a cell concentration of approximately 8×10^8 CFU/ml. The cells were suspended in phosphate-buffered saline, which contained (per liter of distilled water) 0.45 g of KH_2PO_4, 1.16 g of K_2HPO_4, and 8.5 g of NaCl (pH 7.0). Samples were taken 2 weeks after inoculation and every 4 weeks thereafter.

At each sample time five stem sections per plant were excised, weighed, surface sterilized in a solution containing 0.525% sodium hypochlorite and 10.0% ethanol, rinsed in sterile water, and homogenized in 10 ml of phosphate-buffered saline. The homogenate was diluted in 10-fold steps, and plated onto SCM medium, and the preparations were incubated for 7 to 10 days at approximately 27°C. Following incubation, 1,000 representative colonies (100 per plant) were replica plated onto plates containing GC medium or SCM medium supplemented with kanamycin (100 μg/ml), onto plates containing SCM medium supplemented with tetracycline (2 μg/ml), and onto plates containing unamended SCM medium or GC medium so that doubly sensitive colonies were maintained. Colonies were scored for antibiotic resistance or sensitivity after 3 to 5 days of incubation at 27°C.

Field tests performed at our research farm near Ingleside, Md., and at the DeKalb-Pfizer Genetics Research Center, Illiopolis, Ill., served as sources of field-grown corn plants

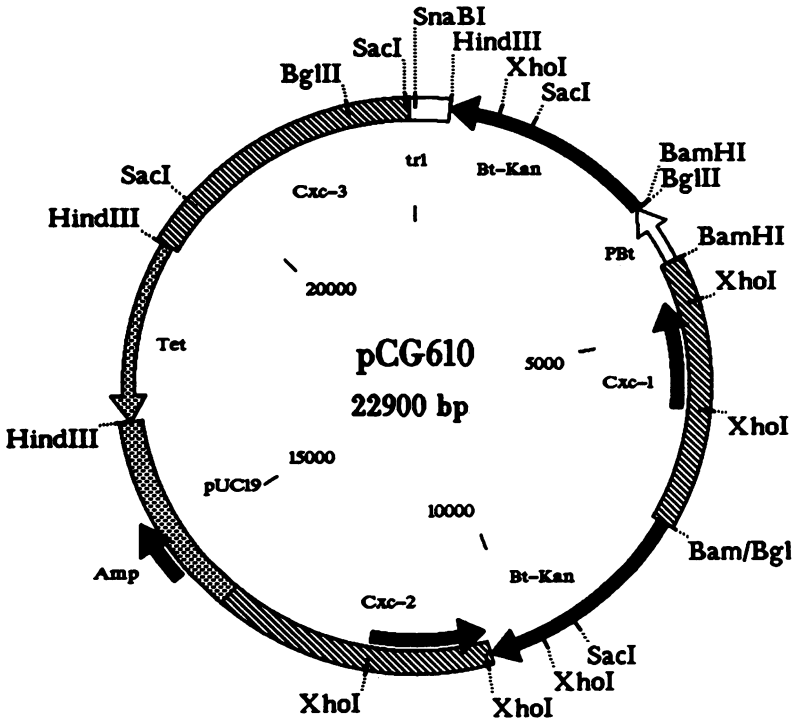

FIG. 1. Physical map of pCG610. Plasmid pCG610 contains single copies of pUC19 and the tetracycline resistance gene from pT181 (Tet) as a 2.3-kb *Hin*dIII fragment (stipled region). There are two copies of the δ-endotoxin gene fusion (Bt-Kan) oriented as inverted repeats (doubly cross-hatched arrows). One copy of the gene fusion contains the native δ-endotoxin promoter (PBt) (open arrow) and the $\lambda t_R 1$ transcription terminator (trl) (open box). A region of Cxc-1 and Cxc-2 are direct repeats of *C. xyli* subsp. *cynodontis* DNA (solid arrows). Singly cross-hatched regions represent *C. xyli* subsp. *cynodontis* genomic DNA. An inversion of the linear genomic DNA has occurred between the *Bgl*II site in Cxc-3 and the *Bgl*II-*Bam*HI junction between Cxc-1 and the promoterless Bt-Kan cassette. The arrows show the direction of transcription from the PBt promoter and the translation of the Amp, Tet, and Bt-Kan genes.

inoculated with *C. xyli* subsp. *cynodontis* MDR1.3. The corn hybrid used at both sites was Dekalb T1100. Seeds were inoculated with strain MDR1.3 prior to planting. The protocol called for seed imbibition, followed by application of a pressure differential in a vessel containing a buffered suspension of the bacteria. The seeds were then removed from the inoculation suspension and dried in a forced air dryer (10a). Ten inoculated plants per sample time per site were destructively sampled. Each resulting homogenate was serially diluted and plated, and colonies were assayed for drug resistance phenotype as described above for Bermuda grass. A subsample of 175 colonies (101 from Illiopolis and 74 from Ingleside) was chosen for an analysis in which DNA-DNA hybridization was used.

In vitro growth and segregation studies. The in vitro growth rates of wild-type strain MDE1, recombinant strain MDR1.3, and segregant strain MDS1.1079 were determined. Quadruplicate 300-ml sidearm flasks containing 50 ml of S-27 medium were inoculated with mid-exponential-phase cells to a density of approximately 5 Klett units (corresponding to an optical density at 660 nm of 0.02 or about 6×10^7 CFU/ml). The growth of each strain was monitored by using a Klett-Summerson photoelectric colorimeter fitted with a red filter. For segregation studies, five replicate culture tubes containing 3 ml of S-27 broth were inoculated with strain MDR1.3. The cultures were grown to late exponential phase, and then 0.1-ml aliquots were used to inoculate fresh medium. At each transfer time, aliquots were diluted and spread plated onto SCM medium, and 100 colonies were analyzed for antibiotic resistance or sensitivity as described above. A total of 50 colonies having the Tet^s Kan^s phenotype were examined for the presence of specific gene sequences by using the dot blot hybridization assay described above.

RESULTS

Description of pCG610. To introduce heterologous DNA into *C. xyli* subsp. *cynodontis* we used plasmid vectors that do not contain *C. xyli* replicons but have the ability to integrate into the *C. xyli* subsp. *cynodontis* chromosome. One such vector is pCG610 (Fig. 1), which comprises (i) pUC19 (24), a replicon that functions in *E. coli* but not in *C. xyli*, thereby providing selection for integration into the *C. xyli* subsp. *cynodontis* chromosome; (ii) a tetracycline resistance gene from *Staphylococcus aureus* plasmid pT181 (described by Kahn and Novick [15]) that is a selectable marker in *C. xyli* subsp. *cynodontis*; (iii) a sequence of *C. xyli* subsp. *cynodontis* DNA to allow integration of the vector through homologous crossing over; and (iv) δ-endotoxin gene sequences inserted into the integration vector, where, as a part of the crossover event, they become part of the *C. xyli* subsp. *cynodontis* chromosome.

The δ-endotoxin gene cassette (Bt-Kan) in pCG610 is an in-frame translation fusion at the *Xho*I site of the *B. thuringiensis* subsp. *kurstaki* HD73 δ-endotoxin gene (1) and the *npt*II gene from TN5 (6), which is able to confer kanamycin resistance to *C. xyli* subsp. *cynodontis*. The fusion includes the first 1,830 bp of the δ-endotoxin gene and the entire 786

bp of the *npt*II gene (5a). Strains of *C. xyli* subsp. *cynodontis* containing pCG610 are resistant to kanamycin at a concentration of 200 μg/ml. The *C. xyli* subsp. *cynodontis* genomic DNA in our integration vectors was originally isolated from a pUC19 library as a 12.5-kb partial *Sau*3AI fragment. Further work resulted in smaller genomic DNA regions and insertion of Bt-Kan cassettes into a variety of sites. Two of these constructions were combined to yield pCG610, which has two copies of the Bt-kan fusion. One copy has no explicit promoter, while the other copy contains the native δ-endotoxin promoter from *B. thuringiensis* (Fig. 1, PBt). Also, the orientation of the incoming *Bgl*II fragment in pCG610 produced an inversion of the *C. xyli* subsp. *cynodontis* DNA relative to the linear map of the genome. This region is bounded by the *Bgl*II site in Cxc-3 and the *Bgl*II-*Bam*HI junction at the end of Cxc-1. Another segment of the *C. xyli* subsp. *cynodontis* DNA was duplicated to generate two direct repeats (Fig. 1, solid arrows).

Structural analysis of pCG610 in *C. xyli* subsp. *cynodontis* transformants. Strain MDR1.3 was made by introducing pCG610 into strain MDE1 by polyethylene glycol-assisted protoplast transformation and selection for tetracycline resistance (10b). The fate of pCG610 in *C. xyli* subsp. *cynodontis* was determined by Southern hybridization. In order to demonstrate that pCG610 had integrated into the chromosome of *C. xyli* subsp. *cynodontis*, genomic DNAs from strains MDE1 and MDR1.3 were digested with several enzymes which either did not cleave pCG610 or cleaved the plasmid at a single site. The hybridization probe was pCG500, a plasmid that contains only pUC19 and the Tet gene. If pCG610 was maintained as an extrachromosomal plasmid, then when the *Sna*BI (single site in pCG610) digest was probed with pCG500, this digest would have yielded a 22.9-kb band corresponding to linearized pCG610. However, if pCG610 was integrated into the chromosome, a larger fragment should have been observed since other sites for *Sna*BI that border the chromosomal insertion are present in the genome of strain MDR1.3. Digestion with *Bam*HI should have yielded similar results as the two *Bam*HI sites in pCG610 are separated by 700 bp. When DNA from strain MDR1.3 was digested with *Sna*BI or *Bam*HI and hybridized with pCG500, fragments that were 33 and 26.5 kb long were generated; for comparison, pCG610 digests yielded 22.9- and 22.2-kb fragments, respectively (Fig. 2). This was the expected result if the plasmid was integrated into the *C. xyli* subsp. *cynodontis* chromosome.

Restriction enzymes which do not cleave pCG610 (*Not*I and *Sfi*I) were also used to determine whether integration had occurred. When genomic DNA from strain MDR1.3 was digested with *Not*I and *Sfi*I, single bands at 33 and 49 kb, respectively, were present after hybridization with pCG500 (Fig. 2). If pCG610 was maintained as an extrachromosomal element, both of these digests, as well as undigested DNA from strain MDR1.3, would have given identical bands. It is also likely that only one chromosomal site was used for a single integration event since multiple copies of pCG610 in strain MDR1.3 would have yielded multiple bands in the *Not*I and *Sfi*I lanes. Integration of tandem copies of pCG610 did not occur as the observed *Not*I fragment (33.0 kb) was too short for two copies of the plasmid (45.8 kb).

Attempts to identify nonintegrated plasmid DNA were carried out by performing Southern hybridization of CsCl-ethidium bromide gradient fractions of strain MDR1.3 total DNA. When nick-translated pCG500 was used as the hybridization probe, no extrachromosomal plasmid forms were detected (data not shown). Southern hybridizations were done with restriction digests of DNA from strain MDR1.3 to determine the integrity of chromosomally inserted pCG610 by using individual probes that represented the entire physical map of pCG610. No rearrangements were detected (data not shown). These results indicated that pCG610 was maintained in the chromosome as a circularly permuted form with flanking repeats.

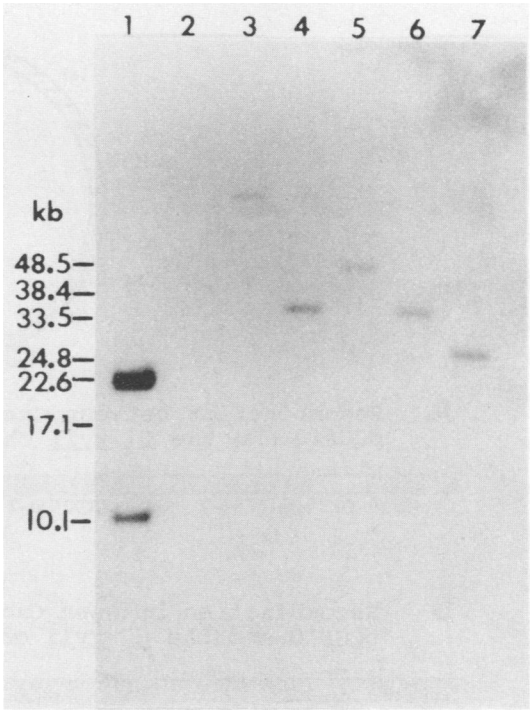

FIG. 2. Southern blot analysis of *C. xyli* subsp. *cynodontis* MDE1 and MDR1.3 to determine integration. DNAs from strains MDE1 and MDR1.3 were digested with *Sna*BI, *Bam*HI, *Not*I, or *Sfi*I, separated on a 0.25% agarose gel, and blotted onto a Nytran membrane. pCG500 (the Tet gene cloned in pUC19) was nick translated with [α-^{32}P]dCTP and used as the hybridization probe. Lane 1, pCG610 digested with *Sna*BI, showing a single linear fragment (22.9 kb) that comigrated with a linear fragment (22.2 kb) from pCG610 digestion with *Bam*HI. pCG610 digested with *Sac*I gave a 10.4-kb fragment that was recognized by the probe; lane 2, strain MDE1 DNA digested with *Not*I; lane 3, undigested strain MDR1.3 DNA; lane 4, strain MDR1.3 DNA digested with *Not*I; lane 5, strain MDR1.3 DNA digested with *Sfi*I; lane 6, strain MDR1.3 DNA digested with *Sna*BI; lane 7, strain MDR1.3 DNA digested with *Bam*HI.

The specific region of DNA in pCG610 involved in the recombination event with the chromosome of strain MDE1 could not be readily determined from the structural analysis of the transformants since multiple sites of homology with the *C. xyli* subsp. *cynodontis* chromosome are present in pCG610. Since Cxc-3 in Fig. 3 was rearranged (inverted) relative to the genomic map during a cloning step in *E. coli*, it is unlikely that this is the site of recombination. Both Cxc-1 and Cxc-2 are possible recombination sites for integration, and the predicted structures are shown in Fig. 3. Further recombination events probably occurred following the initial integration event since the loss of the genetic markers contained within the direct chromosomal repeats could occur by homologous crossover (Fig. 3). Indeed, as shown below, segregation of the drug markers (Tet and Kan) and the δ-endotoxin gene was observed.

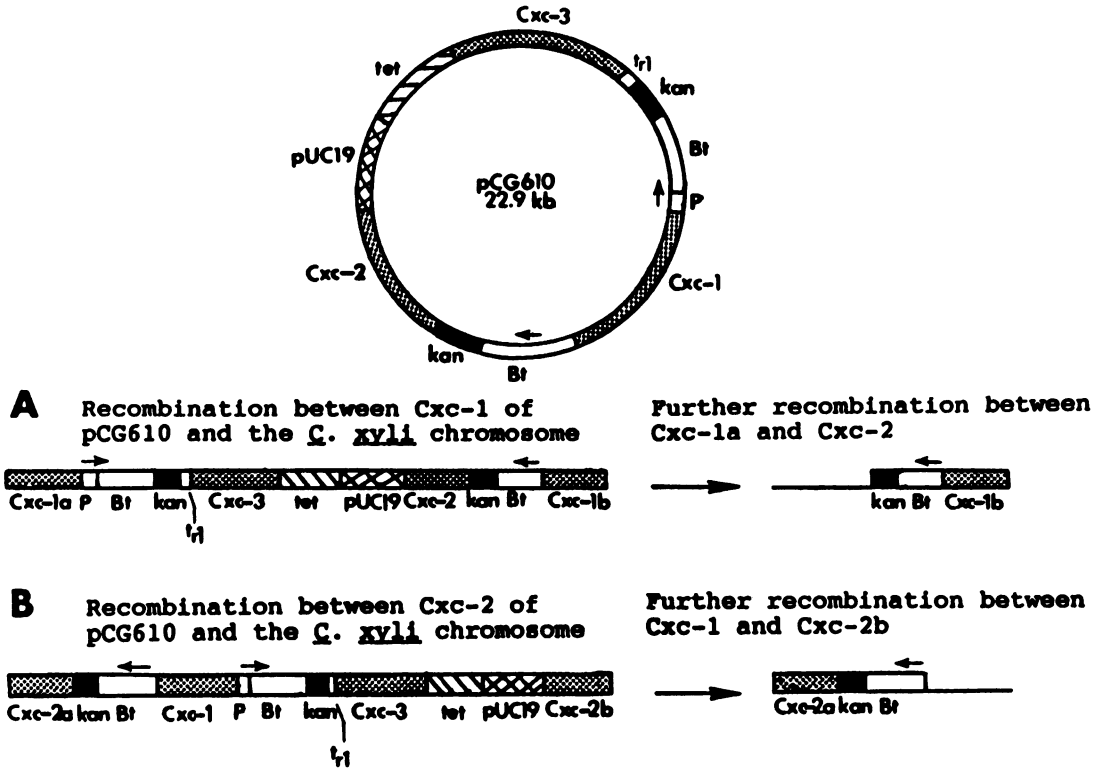

FIG. 3. Integration of pCG610 into the *C. xyli* subsp. *cynodontis* chromosome. A single recombination event in Cxc-1 or Cxc-2 led to two different integrative structures. In either case, further recombination events between *C. xyli* segments resulted in deletions of internal fragments.

In planta segregation studies. Figure 4 shows quantitative data for segregant colonies (colonies lacking antibiotic resistance in a plating assay) isolated from Bermuda grass over time. Because of the large numbers of *C. xyli* subsp. *cynodontis* cells present in Bermuda grass, it was possible to plate plant homogenates that were sufficiently diluted that contaminants were not present on semi-selective SCM medium (9).

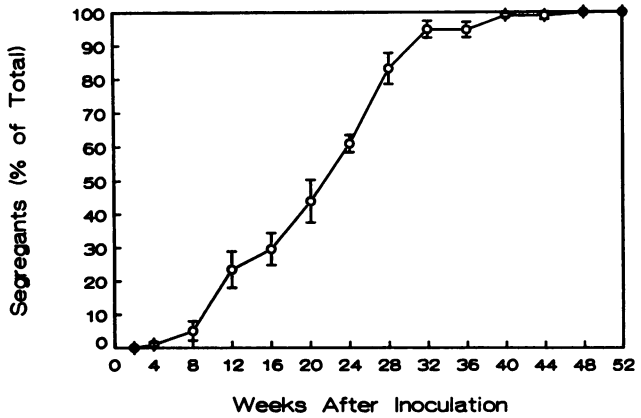

FIG. 4. Incidence of phenotypic segregants (colonies lacking resistance to tetracycline and kanamycin) among *C. xyli* subsp. *cynodontis* colonies isolated from Bermuda grass following inoculation with strain MDR1.3. At each sample time, 1,000 colonies from 10 plants were assayed for antibiotic phenotype. The means and standard errors (bars) are indicated.

Phenotypic segregants were first detected at 4 weeks after inoculation with strain MDR1.3. The segregant proportion (the ratio of segregants to total colonies isolated) increased steadily over the duration of the experiment and began to approach 100% at 32 weeks postinoculation. At the final sampling time less than 0.1% of the colonies recovered retained both the tetracycline and kanamycin resistance genes.

DNA hybridization assays were carried out with randomly selected colonies isolated from Bermuda grass plants. If further recombination occurred after the initial integration event between Cxc-1a and Cxc-1b or Cxc-2a and Cxc-2b (Fig. 3), then all three DNA sequences (Tet, Kan, and δ-endotoxin) would have been eliminated from the chromosome. The DNA hybridization data obtained by using specific probes showed a loss of the resistance genes and the δ-endotoxin gene whenever both antibiotic resistance phenotypes were lost. A total of 702 Tet^s Kan^s isolates were tested over the course of this experiment. Fewer Tet^r Kan^r colonies (85 colonies) were assayed because these colonies were assumed to have retained the genes conferring resistance and because this phenotypic class decreased in frequency over the course of the experiment. Hybridization assays demonstrated that all of the colonies that retained the resistance phenotype contained both resistance genes, as well as the δ-endotoxin gene.

Although not shown in Fig. 3, other recombination events could occur (for example, between Cxc-2 and Cxc-1b or between Cxc-1 and Cxc-2b, leaving the Tet gene and a single copy of the Bt-Kan cassette). The phenotype of the resulting segregants (Tet^r Kan^r) would have been indistinguishable from that of strain MDR1.3. Recombination events were also

TABLE 1. Incidence of phenotypic segregants among colonies isolated from field-grown corn plants that were seed inoculated with *C. xyli* subsp. *cynodontis*

Site	No. of weeks after planting	% of segregants in stem[a]
Ingleside, Md.	2	0[b]
	10	0.3 ± 0.3
	14	10.8 ± 3.4
	18[c]	11.63 ± 6.2
Illiopolis, Ill.	2	0[b]
	11	3.2 ± 1.6
	15	10.7 ± 2.7
	19[c]	12.4 ± 6.9

[a] Percentage of all *C. xyli* subsp. *cynodontis* colonies isolated that were segregants. All other colonies (nonsegregants) retained both antibiotic resistance markers. Mean ± standard error.

[b] Standard errors were not calculated because of a lack of variance in the data.

[c] Approximate harvest times for these fields.

possible between Cxc-1a and Cxc-2 or between Cxc-1 and Cxc-2b, which would have deleted the Tet gene. At 44 weeks postinoculation, the phenotypic class (Tet^s Kan^r) predicted from this event was observed. Of the 1,000 colonies for which phenotypes were determined, 11 had retained kanamycin resistance while losing tetracycline resistance. A hybridization analysis showed that these segregants had lost the tetracycline gene but still contained the kanamycin resistance gene and the δ-endotoxin gene. This phenotypic class was not observed at the last sampling time, 52 weeks after inoculation. The 11 colonies of this class of segregants were rare (0.13%) compared with the 8,273 colonies of the Tet^s Kan^s phenotype observed over the course of the experiment. The Tet^r Kan^s phenotype was never observed.

For field-grown corn plants, segregant colonies were not observed in plants that were sampled 2 weeks after planting, but were observed later in the season (Table 1). The incidence of segregants in the total *C. xyli* subsp. *cynodontis* population increased similarly at the two sites, reaching 12 and 14% for Ingleside and Illiopolis, respectively, by harvest time (>120 days after planting). Hybridization assays revealed that all 96 of the Tet^s Kan^s colonies from Illiopolis and all 69 colonies having the same phenotype from Ingleside contained no DNA homologous to probes encoding resistance to tetracycline or kanamycin or encoding δ-endotoxin. All 10 colonies having the Tet^r Kan^r phenotype (5 from each site) hybridized to all three probes.

In vitro segregation studies. Segregation occurred in vitro and produced curves that were similar in shape (Fig. 5) to the curves obtained for in planta segregation (Fig. 4), although the time required was much shorter. There was a high degree of variation among replicate flasks with respect to when the proportion of segregants began to increase. Segregant frequency began increasing in some flasks as soon as 10 days after inoculation, while in other flasks it took more than 40 days. All of the segregants which we observed had the Tet^s Kan^s phenotype. A total of 50 segregant colonies were selected for DNA hybridization, and we confirmed that all of these colonies had lost both genes for antibiotic resistance and the δ-endotoxin gene.

In vitro growth rates. The instantaneous growth rates and the doubling times for strains MDE1, MDR1.3, and MDS1.1079 (a segregant of MDR1.3 lacking Tet, Kan, and δ-endotoxin) were determined from the linear regions of the growth curves between 19.5 and 25.5 h postinoculation. The

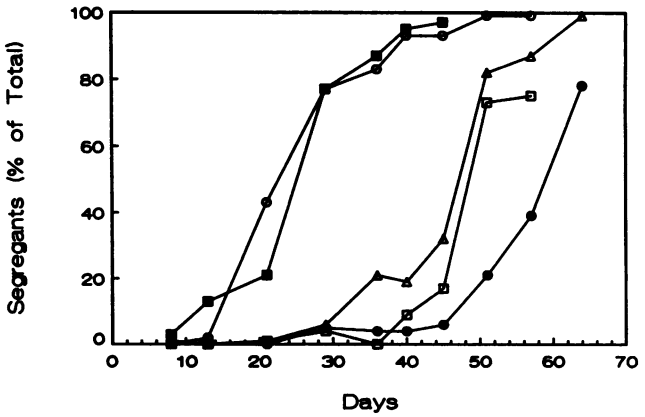

FIG. 5. Incidence of phenotypic segregants (colonies lacking resistance to tetracycline and kanamycin) among *C. xyli* subsp. *cynodontis* colonies recovered over time from five replicate shake flasks (indicated by different symbols) following inoculation with strain MDR1.3.

doubling time for strain MDE1 was 5.5 h, whereas the doubling time for strain MDR1.3 was 7.0 h, which was 21.6% longer than the doubling time for strain MDE1. The doubling time for strain MDS1.1079 was 5.7 h, a value similar to the value for strain MDE1 (Table 2).

DISCUSSION

A recombinant strain of *C. xyli* subsp. *cynodontis* containing chromosomally inserted genes eventually shed these genes when it was grown in vitro or in planta. The deletion usually resulted in the loss of the entire integrated plasmid. The integration event apparently occurred by homologous recombination. If integration of pCG610 into the *C. xyli* subsp. *cynodontis* chromosome occurred by a single crossover at a region of homology, duplication of a portion of the cloned genomic DNA in the vicinity of the crossover would have occurred. Since the duplicated *C. xyli* subsp. *cynodontis* DNA would be arranged as a direct repeat flanking the integrated plasmid sequences, excision by recombination across the region of homology would result in the loss of the integrated DNA.

The dominant segregant class of strain MDR1.3 from shake flask and in planta experiments had lost the entire integrated plasmid from the chromosome. The only other segregant class that was detected had retained at least one copy of the Bt-Kan cassette. This configuration was most likely derived from an internal recombination event as shown in Fig. 3. Since pCG610 contains direct repeats of *C. xyli* subsp. *cynodontis* DNA, it is not surprising that internal recombination occurred. We have developed other vectors

TABLE 2. Comparison of growth rates of *C. xyli* subsp. *cynodontis* strains

Strain	Specific growth rate (h^{-1})[a]	Generation time (h)
MDE1	0.126 ± 0.009	5.5
MDR1.3	0.098 ± 0.003	7.0
MDS1.1079	0.122 ± 0.004	5.7

[a] Specific growth rates are averages from four flasks. Mean ± standard deviation.

that contain only one integrative sequence without direct repeats. Initial studies in which plasmids containing only one integrative sequence were used indicated that markers introduced on these vectors were also lost from the chromosome (16b).

Segregants that have lost all three of the introduced genes gain predominance in the *C. xyli* subsp. *cynodontis* population in Bermuda grass over time. This segregant population could increase as a result of (i) a genetic event which results in gene deletion and (ii) subsequent growth of segregant cells. If the segregant population has a higher growth rate than the recombinant in planta, as has been demonstrated in vitro, then segregant cells would eventually become the dominant type, even though the rate of deletion may be quite low on a per-cell basis. The mathematics of segregation have been described by Cooper et al. (7), who used plasmid-bearing strains of *E. coli*. This test system is biologically different from the one described here, but is mathematically analogous. Sigmoidal curves that show an increase in plasmid-free cells and are similar to the segregation curves which we obtained with strain MDR1.3 were predicted and confirmed experimentally only when the growth rate difference between plasmid-free cells and plasmid-bearing cells was significantly greater than the rate at which plasmid-free cells arose directly from plasmid-bearing cells.

From a product development standpoint, it is critical that the δ-endotoxin protein must be maintained at levels high enough to be efficacious during the growing season. In this study, segregant colonies made up less than 15% of the total *C. xyli* subsp. *cynodontis* colonies isolated from corn at the end of the growing season. The segregation process may serve as an environmental safety feature, in which the recombinant reverts to a strain similar, if not identical, to the wild-type strain. The continual loss of the δ-endotoxin gene reduces the chances of activity against nontarget insects in the unlikely event that the recombinant endophyte escapes from corn to a suitable perennial host.

ACKNOWLEDGMENTS

We thank Amy Stanbury and Angie Ricotta for valuable technical assistance. We also thank Jim Davis and Peter Carlson for critical review of the manuscript.

REFERENCES

1. **Adang, M. J., M. J. Staver, T. A. Rocheleau, J. Leighton, R. F. Barker, and D. V. Thompson.** 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene **36**:289–300.
2. **Aiba, S., Y. Moden, M. Ohnishi, J. Koizumi, and Z. Ming.** 1986. Stabilization in *Bacillus stearothermophilus* of a recombinant plasmid carrying the homologous α-amylase gene. J. Chem. Technol. Biotechnol. **36**:319–328.
3. **Albertini, A. M., M. Hofer, M. P. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29**:319–328.
4. **Ballester, S., P. Lopez, M. Espinosa, J. C. Alonso, and S. A. Lacks.** 1989. Plasmid structural instability associated with pC194 replication functions. J. Bacteriol. **175**:2271–2277.
5. **Ballester, S., P. Lopez, M. Espinosa, and S. A. Lacks.** 1986. Selective advantage of deletions enhancing chloramphenicol acetyltransferase gene expression in *Streptococcus pneumoniae* plasmids. Gene **41**:153–163.

5a. **Barnes, W.** Personal communication.

6. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19**: 327–336.
7. **Cooper, N. S., M. E. Brown, and C. A. Caulcott.** 1987. A mathematical method for analyzing plasmid stability in microorganisms. J. Gen. Microbiol. **133**:1871–1880.
8. **Davis, M. J., and B. J. Angustin.** 1984. Occurrence in Florida of the bacterium that causes bermudagrass stunting disease. Plant Dis. **68**:1095–1097.
9. **Davis, M. J., A. J. Gillaspie, A. K. Vidaver, and R. W. Harris.** 1984. *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria including *Clavibacter xyli* sp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. Int. J. Syst. Bacteriol. **34**:107–117.
10. **Drahos, D. J., B. C. Hemming, and S. McPherson.** 1986. Tracking recombinant organisms in the environment: β-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Bio/Technology **4**:439–444.

10a. **Fahey, J. W., M. B. Dimock, S. F. Tomasino, J. M. Taylor, and P. S. Carlson.** *In* Microbial ecology of leaves, in press.

10b. **Gunyuzlu, P.** Unpublished data.

11. **Hahn, J., and D. Dubnau.** 1985. Analysis of plasmid deletional instability in *Bacillus subtilis*. J. Bacteriol. **162**:1014–1023.
12. **Hass, L. O., J. M. Cregg, and M. A. G. Gleeson.** 1990. Development of an integrative DNA transformation system for the yeast *Candida tropicalis*. J. Bacteriol. **172**:4571–4577.
13. **Jansson, J. K., W. E. Holben, and J. M. Tiedje.** 1989. Detection in soil of a deletion in an engineered DNA sequence by using DNA probes. Appl. Environ. Microbiol. **55**:3022–3025.
14. **Kadam, K. L., K. L. Wollweber, J. C. Grosch, and C. J. Yun.** 1987. Investigation of plasmid instability in amylase-productive *Bacillus subtilis* using continuous culture. Biotechnol. Bioeng. **29**:859–872.
15. **Kahn, S. A., and R. P. Novick.** 1983. Complete nucleotide sequence of pT181, a tetracycline-resistant plasmid from *Staphylococcus aureus*. Plasmid **10**:251–259.
16. **King, R. J., K. A. Short, and R. J. Seidler.** 1991. Assay for detection and enumeration of genetically engineered microorganisms which is based on the activity of a deregulated 2,4-dichlorophenoxyacetate monooxygenase. Appl. Environ. Microbiol. **57**:1790–1792.

16a. **Kostka, S. J., P. W. Reeser, and D. P. Miller.** 1988. Abstr. Annu. Meet. Am. Phytopathol. Soc., abstr. no. 222.

16b. **Lampel, J. S., and J. T. Turner.** Unpublished data.

17. **Liao, C. H., and T. A. Chen.** 1981. Isolation, culture and pathogenicity to sudangrass of a corynebacterium associated with ratoon stunting disease of sugarcane and with bermudagrass. Phytopathology **71**:1303–1306.
18. **Lopez, P., M. Espinosa, B. Greenberg, and S. A. Lacks.** 1984. Generation of deletions in pneumococcal *mal* genes in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81**:5189–5193.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Morgan, J. A. W., C. Winstanley, R. W. Pickup, J. G. Jones, and J. R. Saunders.** 1989. Direct phenotypic and genotypic detection of a recombinant pseudomonad population released into lake water. Appl. Environ. Microbiol. **55**:2537–2544.

20a. **Reeser, P. W., and S. J. Kostka.** 1988. Abstr. Annu. Meet. Am. Phytopathol. Soc., abstr. no. 223.

21. **Shaw, J. J., and C. I. Kado.** 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. Bio/Technology **4**:560–564.
22. **van der Laan, J. C., G. Gerritse, L. J. S. M. Mulleners, R. A. C. van der Hoek, and W. J. Quax.** 1991. Cloning, characterization, and multiple chromosomal integration of a *Bacillus* alkaline protease gene. Appl. Environ. Microbiol. **57**:901–909.
23. **Vidaver, A. K.** 1977. Maintenance of viability and virulence of *Corynebacterium nebraskensense*. Phytopathology **67**:825–827.
24. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.