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Denaturation of Circular or Linear DNA Facilitates Targeted Integrative Transformation of *Streptomyces coelicolor* A3(2): Possible Relevance to Other Organisms

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Using *Streptomyces coelicolor* A3(2) protoplasts, the number of transformants obtained by homologous recombination of incoming double-stranded circular DNA with the recipient chromosome was greatly stimulated by simple denaturation of the donor DNA. This procedure was very effective with inserts over a ca. 100-fold size range, the largest tested being ca. 40-kb inserts in cosmids. These observations led to transformation experiments with linearized cloned DNA and randomly sheared genomic DNA. In both cases, DNA denaturation led to significant levels of transformation. Most of the transformants had resulted from the predicted homologous recombination events. A number of genetic manipulations will be made easier or possible by these procedures.

Streptomyces coelicolor A3(2), genetically the most studied of streptomycetes, serves as a model for the many important antibiotic-producing species of its genus and closely related genera such as *Saccharopolyspora* (10). Its morphologically complex colonies, consisting of a much-branched substrate mycelium and a spore-bearing aerial mycelium, have also attracted analysis. Many genetic tools have been developed for the study of *S. coelicolor* A3(2), including an almost complete ordered cosmid library of chromosomal fragments (32). However, because it typically takes at least 4 days for streptomycete colonies to develop through to abundant sporulation, genetic experiments involving multiple rounds of growth can be protracted. This is often the case in gene disruption and gene replacement (procedures which will become increasingly important after future extensive genome sequencing).

Often, these experiments involve the manipulation of *S. coelicolor* DNA in an *Escherichia coli* plasmid that has no *Streptomyces* origin of replication, followed by introduction of the resulting constructs into *S. coelicolor* by protoplast transformation (22). Successful transformation depends on recombination between the *S. coelicolor* insert and its homolog in the *S. coelicolor* genome. The number of transformants obtained when *S. coelicolor* protoplasts are transformed with such *E. coli* double-stranded plasmids containing *S. coelicolor* DNA is typically rather low (usually in the range of 1 to 100 per μg of plasmid DNA). Because double crossover events of the kind desired for many gene replacements usually (but not always [4, 21]) make up only a minority of transformation events (typically, most are single crossovers, giving rise to integration of the vector), further rounds of growth and replication are often needed to detect the infrequent segregants in which a second crossover has occurred. Increasing the frequency of primary transformants, so that double crossover events could more often be represented at this stage, would thus be a significant benefit.

Hilleman et al. (15) found that single-stranded circular *E. coli* plasmid DNA containing 4 kb of *Streptomyces viridochromogenes* DNA was 10 to 100 times more efficiently rescued in *S. viridochromogenes* by homologous recombination (up to 625 transformants per μg) than was the equivalent double-stranded molecule. To allow preparation of single-stranded DNA (ssDNA), the pBluescript-derived phasmid vector pDH5, containing the coliphage f1 origin of replication and the thiostrepton resistance gene (*tsr*) for selection in *Streptomyces*, was constructed (15). However, application of this useful system is sometimes problematical because the yields of ssDNA with some constructs are very low. This difficulty is compounded when the recipient is to be *S. coelicolor* A3(2), which contains a powerful methyl-sensing restriction system (22) that appears to be effective even on ssDNA (16), necessitating the use of a nonmethylating *E. coli* host adapted for ssDNA preparation (16, 22, 24, 25). Moreover, for other purposes there is often a need to transform *S. coelicolor* with *E. coli* plasmids other than phasmids such as pDH5. A case in point is the direct screening of *E. coli* cosmids containing *S. coelicolor* DNA for ability to complement *S. coelicolor* mutants (30, 32).

We therefore sought methods for further increasing the yield of integration events that were applicable to a wider range of situations. This led to the discovery that transformation of *S. coelicolor* dependent on homologous recombination is strongly stimulated by denaturation of double-stranded circular DNA. Remarkably, significant numbers of transformants were also obtained when the denaturation procedure was applied to linear DNA. We speculate that denaturation of DNA may stimulate targeted integrative transformation of other organisms.

MATERIALS AND METHODS

Strains and growth conditions. *S. coelicolor* A3(2) strains used were J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$SCP2$^-$) (9) and the J1501 derivative J2220, in which a 0.6-kb *Bgl*II-*Bst*EII, *whiB*-containing fragment of the chromosome (11) was replaced by a cassette containing the hygromycin resistance gene (*hyg*) of *Streptomyces hygroscopicus* (36) and the glucose kinase gene (*glkA*) of *S. coelicolor* A3(2) (1, 19). This cassette was constructed and provided by J. M. Fernández-Abalos. *E. coli* strains used were DH5α (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* ϕ80 d*lacZ*Δ*M15*) (14, 34), SURE {e14$^-$ Δ(*mcrCB-hsdSMR-mrr*)171 *endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbsC umuC*::Tn*5 uvrC* [F′ *proAB laqI*^{q} *lacZ*Δ*M15* Tn*10* Amy Cam^r; Stratagene}, used as a host for stable maintenance of cosmids (13), and ET12567 (F$^-$ *dam13*::Tn*9 dcm6 hsdM hsdR recF143 zjj201*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*) (25) (kindly provided by D. MacNeil, Merck Sharp and Dohme Research Laboratories, Rahway, N.J.), which was used to

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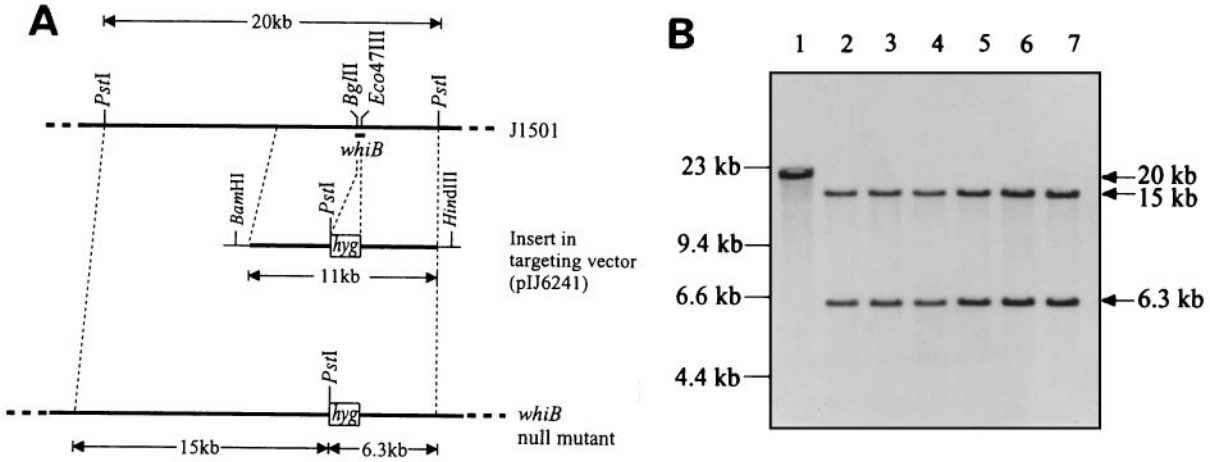

FIG. 1. Verification of replacement of *whiB* by double crossover events following transformation by denatured circular pIJ6241. (A) Relevant features of genomic organization. (B) Analysis of DNA of six putative double crossover recombinants (tracks 2 to 7) by Southern blotting. Track 1 contained DNA from the parental strain J1501. All samples were digested with *Pst*I. The probe was the targeting vector pIJ6241.

produce nonmethylated DNA. Media and culture conditions were as in manuals for *Streptomyces* (18) and *E. coli* (34) genetics. As necessary, antibiotics were added to the following final concentrations: carbenicillin (Link Pharmaceuticals Ltd., Horsham, United Kingdom), 200 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 200 μg/ml; and tetracycline, 10 μg/ml (all Sigma Chemicals Co., St. Louis, Mo.); hygromycin (a gift from the Eli Lilly Co., Indianapolis, Ind.), 150 μg/ml; and thiostrepton (a gift from S. J. Lucania, E. R. Squibb and Sons, Princeton, N.J.), 50 μg/ml.

Plasmids. The *E. coli* vector pDH5 (15) contains *bla* for carbenicillin resistance selection in *E. coli* and *tsr* for thiostrepton resistance selection in *S. coelicolor*. pDH5 has no origin for replication in *Streptomyces*. The bifunctional plasmid pIJ699 (23), capable of replicating in *E. coli* and *S. coelicolor* A3(2), was also used. Ten cosmids containing *S. coelicolor* DNA inserts that hybridized to a *whiB* probe were obtained from the library of Redenbach et al. (32). This library was constructed in a cosmid (Supercos-1) (12) that contains a *neo* gene that confers kanamycin resistance on streptomycetes. Cosmid E112 was selected for further work as one of four containing an intact 20-kb *whiB*-containing *Pst*I fragment (the others were E34, E67, and E69). Cosmids with only part of the fragment were E40, E43, E103, E113, E115, and E116.

DNA preparation. Unless stated otherwise, the nonmethylating strain ET12567 was used as the host for plasmid preparation because of the severe restriction of methylated DNA by *S. coelicolor* A3(2). Plasmids were prepared by alkaline lysis (34). For large-scale preparations, the alkaline lysis step was followed by CsCl-ethidium bromide centrifugation (34). To rescue cosmids present in a free form in *S. coelicolor* (as low-abundance loop-outs from the chromosomally integrated form), the following protocol was used. Spores (ca. 10^8) were inoculated into 50 ml of YEME medium (18) (without kanamycin) and incubated on a rotary shaker (30°C, 40 h). The culture was used to prepare plasmid DNA by alkaline lysis (final volume, 100 μl), and 5-μl samples were used to transform *E. coli* DH5α or ET12567 to kanamycin resistance. To isolate total DNA from *S. coelicolor* derivatives, the procedure of Pospiech and Neumann (31) was used. To purify large electrophoretically separated DNA fragments from agarose gels, QIAEXII (Qiagen) was used as recommended by the manufacturer.

DNA denaturation. Double-stranded DNA (dsDNA) was denatured by heat or alkaline treatment (using the US 70999 kit supplied by U.S. Biochemical, Cleveland, Ohio) as follows.

(i) Glycol-heat treatment. After thorough mixing of DNA (in a total volume of 8 μl of water) with 5 μl of plasmid-denaturing reagent (10 mM Tris HCl [pH 7.5], 1 mM EDTA, 50% glycerol, 50% ethylene glycol) in a microcentrifuge tube, the mixture was incubated at 90 to 100°C for 5 min and then chilled rapidly on ice.

(ii) Alkali treatment. DNA (in a total volume of 9 μl of H_2O) was mixed with 2 μl of 1 M NaOH in a microcentrifuge tube (by tapping the tube and brief centrifugation). Then the mixture was incubated at 37°C for 10 min and rapidly chilled on ice, after which 2 μl of 1 M HCl was added.

Transformation. Transformation of *S. coelicolor* protoplasts in the presence of polyethylene glycol 1000 was done as described previously (18) except that the final wash after dilution of the transformed protoplasts was omitted. We emphasize that the P buffer (50 to 100 μl) in which the protoplasts were suspended prior to addition of denatured DNA protects the protoplasts against inadvertent departure from neutral pH in the DNA solution. A variation of the RbCl-$CaCl_2$ method (14) was used to prepare and transform *E. coli* competent cells (31a).

Southern blotting. A standard method (18) was used for Southern blotting to Hybond N membranes (Amersham). Probes were nonradioactively labelled with digoxigenin, using the DIG DNA labelling kit of Boehringer Mannheim. Hybridization was in stringent conditions (68°C, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate], 0.1% sodium dodecyl sulfate).

Morphological analysis. Aerial mycelium was examined by phase-contrast microscopy of impression preparations (7) of colonies grown for 4 to 6 days on minimal medium (18) supplemented with histidine and uracil, and with glucose replaced by mannitol as the carbon source.

RESULTS AND DISCUSSION

Establishment of a test system. The direct stimulus for the experiments reported here was an interest in *whiB*, a gene specifically needed for sporulation in the aerial mycelium (7, 11). To carry out intensive mutational analysis of *whiB*, more efficient methods for chromosomal gene replacement were desirable. To establish these, we designed a test system based on *whiB* and exploited the white aerial mycelium phenotype of *whiB* mutants (compared with the grey of mature, sporulated wild-type aerial mycelium [7, 17]). Using a construct (pIJ6241 [Fig. 1A]) containing a ca. 9-kb fragment of the *S. coelicolor* chromosome in which most of the centrally located *whiB* gene was replaced by a selectable *hyg* cassette, up to about 10 hygromycin-resistant (Hyg^r) primary transformants of the test host J1501 were obtained per μg of nonmethylated dsDNA. Of the 37 colonies tested, 31 were grey and thiostrepton resistant ($Thio^r$, vector marker), but 6 were white and $Thio^s$. These were the expected phenotypes for single and double crossover classes of recombinants, respectively. The aerial mycelium phenotype observed by phase-contrast microscopy was as described previously for a *whiB* point mutant and for a deletion mutant removing *whiB* and at least part of a downstream gene (7, 11).

Denaturation of plasmid DNA stimulates integrative transformation but depresses transformation by an autonomously replicating plasmid vector. Because ssDNA prepared from recombinant M13 phage particles is particularly efficient in *Streptomyces* transformations that involve homologous recombination (15, 16), we explored the effect of denaturing double-stranded pIJ6241 DNA. The very simple and rapid heat or alkaline denaturation procedures used for preparing DNA sequencing templates from double-stranded plasmid DNA were used. Both proved reproducible and highly effective, typically giving about 100-fold more transformants than dsDNA when J1501 was transformed with nonmethylated pIJ6241 (Table 1). Double crossover events were frequent (ca. 40% of all tested

TABLE 1. Use of alkaline denaturation stimulates transformation of *S. coelicolor* J1501 by pIJ6241[a]

Expt	No. of transformants	
	Denatured pIJ6241[b]	Untreated pIJ6241
1[c]	297, 318	1, 0
2	793, 911	3, 4
3	429, 504	8, 9

[a] In each of three independent experiments, 3.8 μg of pIJ6241, with or without alkali treatment, was used to transform J1501 protoplasts. From each transformation mixture (final volume, 5 ml), two samples of 1 ml were spread on regeneration plates. Hygromycin was added to 150 μg/ml after 16 h. Similar results (not shown) were obtained when heat was used instead of alkali to denature pIJ6241.

[b] Of 149 colonies tested further, 59 showed the expected double crossover phenotype (white, Thio^s) and 83 showed the expected single crossover phenotype (grey, Thio^r). Seven showed an unexpected white, Thio^r phenotype. Southern blotting was used to verify for six white, Thio^s colonies that they had the correct DNA organization for double crossover transformants (Fig. 1B).

[c] In this experiment, controls in which the NaOH and HCl were mixed before addition to the DNA yielded five and four colonies on transformation plates. This showed that the massive stimulation by the denaturation procedure was not a high-salt effect.

progeny). After this initial success, colleagues tested the procedure on genes from various regions of the chromosome, with similar positive results (6, 35). Summarizing their results, the overall transformation frequency was observed to be lower with shorter fragments, but strong stimulation was always obtained after denaturation; often, denatured DNA readily produced transformants in experiments that had failed with the conventional procedure. The smallest fragment tested was 600 bp. Other *S. coelicolor* A3(2) derivatives (the plasmid-free prototrophs M600 [5] and M145 [18]) were also tested as hosts; in both cases, denaturation again caused strong stimulation of integrative transformation, though the precise factor was not determined because both strains gave markedly fewer transformants than J1501 in all conditions (often none or only a few in the absence of denaturation).

The stimulatory effect was specific for insert-directed integrative transformation. When unmethylated pIJ699, a bifunctional plasmid carrying replication origins for both *E. coli* and *S. coelicolor*, was used to transform J1501, the number of Thio^r transformants was reduced about threefold by denaturing treatments.

Alkaline denaturation very strongly stimulates integrative transformation of *S. coelicolor* by cosmids containing *S. coelicolor* DNA. Redenbach et al. (32) recently prepared and ordered a library of *S. coelicolor* DNA inserts in the *E. coli* cosmid Supercos-1, which contains a kanamycin resistance gene (*neo*) selectable in *S. coelicolor*. This has made it feasible to use cosmids from an appropriate region of the genome to transform strains carrying mutations of known map location. Such map-based cloning has been effective for several genes, with transformation frequencies usually in the range 10 to 100/μg of unmethylated native dsDNA (30, 32). If obtainable, higher transformation frequencies might extend the technical possibilities further. For example, unmapped mutations might be complemented by a particular cosmid in a large pool of cosmids. Indeed, we found that alkaline denaturation of unmethylated cosmid E112, which contains *whiB*, reproducibly stimulated transformation of the *whiB* disruption mutant J2200 about 200-fold, transformations usually yielding almost confluent kanamycin-resistant colonies (more than 10^4 colonies per μg, compared with about 50 per μg with native DNA [Fig. 2]). However, detectable transformation was eliminated when cosmid DNA was heat denatured. Presumably the very large size of cosmids (ca. 50 kb) causes differences between the two procedures in the extent of denaturation or renaturation.

Second crossover events in the J2220/E112 transformants should liberate circular DNA, corresponding either to E112 or, if the second crossover is appropriately placed, to E112 with *hyg-glk* replacing *whiB* (Fig. 3A). If such loop-out events were frequent enough, application of a plasmid miniprep procedure to the transformants should yield DNA able to transform *E. coli* strains to carbenicillin resistance, allowing recovery of E112 and its *hyg-glk* derivative, as was reported for *Streptomyces avermitilis* (25, 26). We found that this worked well. For example, using 5-μl samples of 100-μl plasmid preparations from 50-ml cultures of J2220/E112, we obtained 20 transformants with DH5α and 6 with ET12567. Plasmids were reisolated from the *E. coli* transformants and analyzed by *Pst*I digestion (Fig. 3B). Overall, more than half were identical to cosmid E112, but a significant number differed in the way expected if they had picked up the *hyg-glk* cassette from the J2220 chromosome. Only 1 of 23 plasmids examined showed an unpredicted *Pst*I digestion pattern (Fig. 3B, track 6). These results showed that the cosmids had generally integrated into, and excised from, the J2220 chromosome in the predicted manner. Thus, the denaturation procedure did not lead to significant numbers of rearrangements.

Denaturation of methylated DNA does not circumvent the methyl-sensing restriction system of *S. coelicolor* A3(2). Most of the well-known restriction systems are inactive on ssDNA. Although there is evidence that ssDNA prepared from pDH5 derivatives by using helper phage does not bypass the methyl-sensing restriction system of *S. coelicolor* A3(2) (16), it was possible that restriction might be circumvented by the use of denatured DNA. We tested this for both replicative and integrative transformation. First we tried to transform J1501 with methylated DNA of the bifunctional plasmid pIJ699 isolated

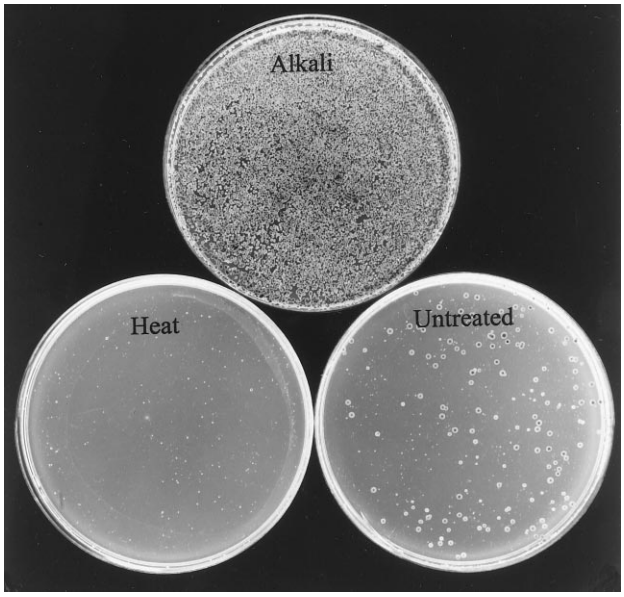

FIG. 2. Transformation of *S. coelicolor* J1501 by a cosmid containing *S. coelicolor* DNA is increased following alkali denaturation of DNA but decreased after heat denaturation. Cosmid E112 containing *whiB* (Fig. 3A) was propagated in *E. coli* ET12567, and 3-μg samples of the unmethylated DNA, treated as shown, were used to transform strain J2220 (in which *whiB* has been replaced by a *hyg-glk* cassette [Fig. 3A]). Half of each transformation mixture was spread on each of two R2YE plates. Kanamycin (1 ml of a 4-mg/ml stock solution; final concentration, ca. 200 μg/ml) was added after 16 h.

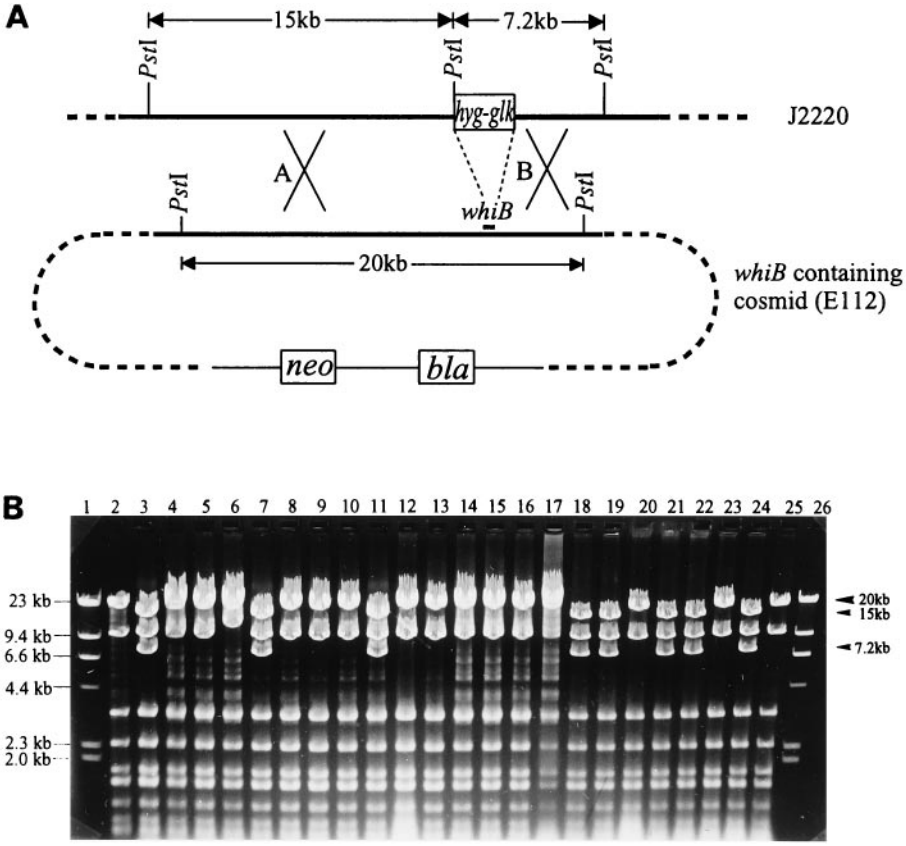

FIG. 3. Recovery of recombinant cosmids after transformation of J2220 by cosmid E112. (A) Relevant features of the *whiB* region of J2220 and of the *whiB*-containing cosmid E112. Dashed lines indicate the uncharacterized sequences flanking the 20-kb *Pst*I fragment. (B) *Pst*I digests of plasmid DNA isolated from carbenicillin-resistant derivatives of *E. coli* DH5α (tracks 3 to 17) or ET12567 (tracks 18 to 25) obtained after transformation with DNA prepared by the plasmid miniprep procedure from a population of J2220 transformants containing integrated cosmid E112. Track 2, digest of original cosmid E112 DNA. All fragments of less than 4 kb are from the insert in the cosmid, but they are located outside the region being considered here and therefore show no changes in pattern. Large arrowhead, position of the 20-kb wild-type *Pst*I fragment containing *whiB*; small arrowheads, positions of 15- and 7.2-kb *Pst*I fragments from the *whiB* region containing the *hyg-glk* cassette. Only one example of an unexpected cosmid structure (track 6) was found.

from *E. coli* DH5α. No transformants (i.e., at least a 10^4-fold reduction compared with an equivalent amount of unmethylated denatured DNA) were obtained with 1 μg of DNA, irrespective of denaturation by heat or alkali. We also tested whether methylated cosmid DNA (isolated from *E. coli* SURE) could transform *S. coelicolor* after alkaline denaturation. No transformants were obtained in conditions that gave nearly confluent (ca. 10^4) colonies with an equivalent amount of unmethylated DNA. These results verified that the *S. coelicolor* restriction system cannot readily be bypassed by the use of ssDNA.

Transformation with linearized cloned DNA. The striking increase in insert-directed integrative transformation brought about by denaturation of plasmids led us to wonder whether denatured linear DNA might also be effective in transformation. Surprisingly, there appear to be no published reports of attempts to transform streptomycetes with cloned linearized DNA, so it was not excluded that even nondenatured linear DNA might be effective in transformation. Indeed, preliminary attempts to transform J1501 with double-stranded pIJ6241 DNA after linearization with various restriction enzymes revealed a low frequency of transformants. To analyze this further, we digested unmethylated pIJ6241 with *Bam*HI plus *Hin*dIII to remove pDH5 containing *tsr* and gel purified the 11-kb *hyg*-disrupted *whiB* fragment. Different amounts of this DNA were used, with or without alkali denaturation, to transform *S. coelicolor* J1501 to hygromycin resistance (Table 2). In every case, a few transformants were obtained. Alkali treatment of the DNA generally stimulated transformation, though by no more than a fewfold. All transformants were Thio^s, as expected, and most of them showed the white (*whiB* mutant) phenotype. About 20% of the transformants obtained with denatured DNA were grey (*whiB*$^+$). Southern blotting of DNA from two white and two grey colonies (Fig. 4) revealed that the

TABLE 2. Transformation of *S. coelicolor* J1501 with linearized pIJ6241 DNA

Amt (ng) of DNA[a]	Alkaline denaturation	No. of Hyg^r transformants with indicated phenotype[b]		Total no. Hyg^r
		White, Thio^s	Grey, Thio^s	
200	No	2	0	2
	Yes	25	8	33
450	No	34	0	34
	Yes	30	5	35
	No	17	0	17
	Yes	41	5	46

[a] Gel-purified *Bam*HI-*Hin*dIII insert from unmethylated pIJ6241 (Fig. 1A).
[b] No Thio^r transformants were obtained.

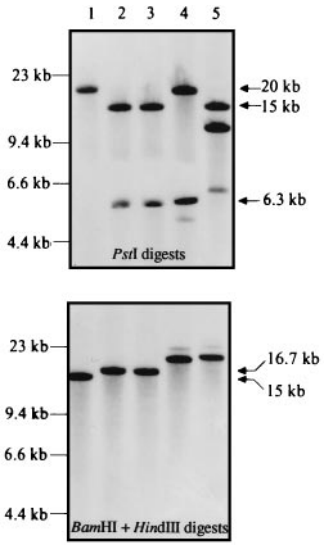

FIG. 4. Hybridization analysis of Southern blots of DNA from transformants of J1501 obtained with purified linear DNA (*Bam*HI-*Hin*dIII insert) from pIJ6241. The probe was pIJ6241. Track 1, J1501 DNA; tracks 2 and 3, DNA from white Hyg^r transformants obtained with undenatured (track 2) or denatured (track 3) DNA; tracks 4 and 5, DNA from grey Hyg^r transformants obtained with denatured DNA. The positions of molecular weight markers are shown at the left, and the arrows on the right side show the sizes of bands important for the analysis (see also Fig. 1).

white colonies had arisen from the expected double crossover events and that the grey colonies had arisen from aberrant integration events in which one of the two crossovers had occurred in an unexpected position, giving rise to a partial duplication. In each case, the second crossover was in a different position, though the resulting duplications both encompassed about 5 kb.

Transformation with genomic DNA preparations. Since cloned linear DNA was effective in transformation, we also tested conventionally prepared total genomic DNA of *S. coelicolor*. DNA isolated from four independent *whiB*::*hyg* transformants was used in attempts to transform J1501, with and without alkaline denaturation. Ostensibly, 4 μg of DNA was used in each experiment, but the optical densities at 260 nm used as a guide were unreliable because considerable levels of RNA and protein remain in DNA prepared by the Pospiech and Neumann (31) procedure. Probably, the actual amount of DNA used was 1 to 2 μg per transformation. The transformations with denatured DNA yielded 53, 78, 44, and 39 Hyg^r colonies. No such colonies were obtained with equivalent amounts of undenatured DNA. All of the Hyg^r transformants showed the white colony phenotype expected from double crossover replacement of the *whiB*$^+$ region by the *whiB*::*hyg* mutation, and Southern blotting using the same restriction enzyme and probe as were used for Fig. 1 confirmed that each of five DNA samples from different transformants contained the expected chromosome organization in the *whiB* region.

Practical significance of these results. (i) Gene replacement in *S. coelicolor*. The use of denaturing procedures on plasmid DNA makes it straightforward and quick to carry out single-step gene replacements. With flanking sequences down to 1 kb, at least several percent of the transformants arise from double crossovers. Since hundreds of transformants are readily obtainable with J1501 (other strains may give fewer transformants), simple replica plating is sufficient to identify the desired recombinants. Because of the high reliability of the procedure, situations in which the lethality of a replacement prevents the production of viable double crossover recombinants will be easier to recognize.

The discovery that cloned linear DNA can transform *S. coelicolor* at a moderate frequency provides an even shorter route to gene replacement, since most primary transformants appear to result from simple double crossover replacements, in which case the time from carrying out a transformation to the first manipulations with the desired transformants may be less than 1 week. The current limitations of transformation with cloned linear DNA are that it has been tested only with comparatively long flanking regions (4 to 5 kb on each side of the *hyg* marker) and that rather low numbers of transformants have been obtained, which puts a higher premium on the use of transformation conditions that are close to optimal.

The effectiveness of transformation with denatured total genomic DNA preparations should make it very straightforward to move gene disruptions, once constructed in the *S. coelicolor* genome, between strains with different genetic backgrounds. It will also make it easy to verify linkage of mutant phenotypes to genes disrupted by resistance genes, either artificially or by transposon mutagenesis. Previous reports of transformation of *Streptomyces* spp. by chromosomal DNA preparations do not appear to have been followed up (20, 27, 29).

(ii) Screening libraries of DNA constructed in vectors such as Supercos-1 by complementation of *S. coelicolor* mutants. Since thousands of kanamycin-resistant transformants are easily obtained when *S. coelicolor* is transformed with denatured, nonmethylated cosmids from the library of Redenbach et al. (32), it becomes possible to screen the entire ordered 319-cosmid library for the ability to complement any mutant, even for an entirely unmapped gene and even if the screening process requires limited individual testing of colonies. [Although the restriction system of *S. coelicolor* A3(2) necessitates passage of the cosmids through a nonmethylating host, this is not a major obstacle.] Thus, pools of, say, 20 cosmids (i.e., 16 pools) could initially be used, positive results being followed up by tests on individual cosmids. Alternatively, plasmid DNA from complemented transformants obtained with a pool can be used to transform *E. coli* directly to rescue the naturally excised cosmid.

(iii) Analysis of subclones for complementation of mutants. The high transformation frequencies obtained with denatured pDH5 derivatives will further encourage the use of subclones constructed in this or similar vectors to localize genes of interest. For example, after identification of a cosmid by mutant complementation, pools of smaller subclones constructed in pDH5 can be passaged through a nonmethylating *E. coli* strain and used to transform the appropriate *S. coelicolor* mutant. Recovery of relevant plasmid DNA could be done as described in references 25 and 26 (e.g., Fig. 3) or by in vitro excision from chromosomal DNA followed by ligation and transformation of *E. coli.*

(iv) Efficient transformation facilitates mutational cloning. In principle, it is possible to generate mutants by using nonreplicating vectors containing random fragments of DNA small enough to fall entirely within the transcription units to be studied (mutational cloning [8]). The isolation of the vector and flanking sequences, as outlined above, then gives rapid access to the desired DNA. However, many genes are monocistronic and less than 1 kb in length, and DNA fragments of no more than a few hundred base pairs would therefore be needed for the successful comprehensive application of this approach. Such small fragments usually give very few transformants by insert-directed recombination in *S. coelicolor*. The

increased integrative transformation brought about by denaturation should facilitate a mutational cloning approach to both primary cloning and subcloning.

(v) Use in mutagenesis. The denaturation method has now allowed us to achieve our initial objective of obtaining thousands of integrative transformants with independently mutagenized *whiB*-containing inserts, thus facilitating extensive mutational analysis.

(vi) Potential utility beyond *S. coelicolor* A3(2). It is highly likely that the methodological improvements reported here for *S. coelicolor* will be readily applicable to any streptomycete capable of accepting DNA from *E. coli*. This group includes many producers of agro-industrially or medically important secondary metabolites. Furthermore, the denaturation method may provide a means of increasing the targeted integration of DNA into organisms such as slow-growing mycobacteria in which the predominance of illegitimate integration events makes it difficult to carry out gene replacement or disruption (2, 3, 28, 33).

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