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Plasmid Marker Rescue Transformation Proceeds by Breakage-Reunion in *Bacillus subtilis*

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Bacillus subtilis **carrying a plasmid which replicates with a copy number of about 1 was transformed with linearized homologous plasmid DNA labeled with the heavy isotopes ^{2}H and ^{15}N, in the presence of $^{32}P_i$ and 6-(*p*-hydroxyphenylazo)-uracil to inhibit DNA replication. Plasmid DNA was isolated from the transformed culture and fractionated in cesium chloride density gradients. The distribution of total and donor plasmid DNA was examined, using specific hybridization probes. The synthesis of new DNA, associated with the integration of donor moiety, was also monitored. Donor-specific sequences were present at a density intermediate between that of light and hybrid DNA. This recombinant DNA represented 1.4% of total plasmid DNA. The latter value corresponded well with the transforming activity (1.7%) obtained for the donor marker. Newly synthesized material associated with plasmid DNA at the recombinant density amounted to a minor portion of the recombinant plasmid DNA. These data suggest that, like chromosomal transformation, plasmid marker rescue transformation does not require replication for the integration of donor markers and, also like chromosomal transformation, proceeds by a breakage-reunion mechanism. The extent of donor DNA replacement of recipient DNA per plasmid molecule of 54 kilobases (27 kilobase pairs) was estimated as 16 kilobases.**

Plasmid transformation in competent cells of *Bacillus subtilis* requires multimeric DNA (6). However, linear or circular monomers can transform efficiently when the recipients carry a plasmid having base sequence homology with the donor DNA (8, 34). This phenomenon has been referred to as plasmid marker rescue transformation (PMRT).

In a number of bacterial systems, *Bacillus subtilis* (2–4, 7, 30; Z. Lorkiewicz, Z. Opara-Kubinska, and W. Szybalski, Fed. Proc. **20**:360, 1961), *Streptococcus pneumoniae* (19), and *Haemophilus influenzae* (29), chromosomal transforming DNA has been shown to be physically integrated into the genomes of the recipient bacteria. In *B. subtilis*, as in the other systems, the product of recombination is a heteroduplex, in which a single-stranded donor fragment is paired to a complementary resident strand (13, 17, 18). The presence of donor DNA atoms in recombinant molecules has led to the generally accepted conclusion that chromosomal transformation (CT) proceeds by a breakage-reunion mechanism.

We have utilized a PMRT system to study recombination in *B. subtilis*. This system is similar to CT in its requirement for homology, *recE*$^{+}$ dependence, and first-order dependence on DNA concentration, in its dependence on donor molecular weight, and in the relationship between physical and genetic distances (8, 34). However, PMRT may conceivably proceed by a copy choice mechanism. In this case, newly synthesized DNA would contribute extensively to the establishment of the recombinant molecule.

The experiments described here were aimed at determining the relative contributions of newly synthesized DNA, as well as old donor and recipient DNA atoms, to the formation of recombinant plasmid DNA in PMRT in *B. subtilis* to distinguish between breakage-reunion and copy choice recombinational mechanisms.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Competent strain BD630 (*leu metB5 hisH2*) carrying plasmid pBD237 (Δ*kan-1 cat*$^{+}$ Δ*hisH1 ermC*$^{+}$) was used as the recipient for transformation, and strain BD910 carrying plasmid pBD235 (*kan*$^{+}$ *cat-1 hisH*$^{+}$ *ermC*$^{+}$) was used for the isolation of donor plasmid DNA. pBD237 and -235 replicate in *B. subtilis* with copy numbers of about 1 (34).

Isolation of plasmid DNA. ^{2}H,^{15}N-labeled and light donor plasmid DNAs were extracted as described previously (21), followed by CsCl-ethidium bromide density gradient centrifugation, as described for *B. subtilis* (20). The heavy labeling medium was essentially that of Dubnau and Davidoff-Abelson (17) except that deuterated algal whole hydrolysate (Merck & Co., Canada) was substituted for the deuterated sugar and amino acid extract in the medium. Standard preparations of ^{2}H,^{15}N,^{3}H-labeled and light ^{3}H-labeled plasmid DNA were prepared as above, except that [^{3}H]thymine (4 μCi/ml) (50 Ci/mmol; Moravek Biochemical) and 250 mg of deoxyadenosine per ml were added during growth in the ^{3}H- and ^{15}N-containing medium to yield plasmid DNA of specific activity 2×10^{4} cpm/μg.

Preparation of competent cells, plasmid transformation, and isolation of donor-recipient complex. Competent cells were prepared as described in reference 17 and separated by the procedure described by Joenje et al. (25), using Renografin (E. R. Squibb & Sons). The competent cell fraction from a 1-liter culture was washed twice in low-phosphate medium and suspended in 24 ml of the same medium containing $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.5 mM). Low-phosphate medium is identical to Spizizen minimal medium (1) but contains 2 mM K_2HPO_4 and 25 mM Tris hydrochloride, pH 7.5, instead of phosphate buffer. An 8-ml amount of this suspension was exposed to ^{2}H,^{15}N-labeled donor DNA, at concentrations of 1 to 2 μg/ml, in the presence of 300 μM 6-(*p*-hydroxyphenylazo)-uracil to inhibit DNA polymerase

III (5). Before transformation, the donor DNA (27 kilobase pairs [kbp]) was cut with restriction endonuclease *StuI* (Boehringer Mannheim Biochemicals) as described previously (34), generating two fragments. The major fragment (24 kbp) carries the transforming markers. The smaller fragment contains sequences essential for plasmid replication. After incubation at 37°C for 20 min, an equal volume of competence medium containing 100 μg of DNase per ml was added, and incubation was continued for 5 min. The cells were then washed twice in Spizizen minimal medium, and plasmid DNA was isolated and analyzed as described below. A small sample was plated for His^+ transformants and for viable count. A 4-ml sample of competent cells was transformed in an identical way except in the presence of 1 mCi of $H_3{}^{32}PO_4$ (ICN) per ml. Plasmid DNA was isolated from this sample and analyzed as described below. To determine specific activities of the deoxyribonucleotide triphosphate pools, 12 ml of competent cell suspension was transformed as above but in the presence of 1 mCi of $H_3{}^{32}PO_4$(ICN) per ml. After 5, 10, and 20 min at 37°C, 4-ml samples were added to equal volumes of competence medium containing 100 μg of DNase (type I; Sigma Chemical Co.) per ml, and incubation was continued for 5 min at 37°C, after which samples were removed. The samples were washed twice and analyzed for specific activity as described below. Plasmid DNA was isolated from the transformed radioactive suspension and from the nonradioactive suspension as described previously (20), and both preparations were purified by passage through an Elutip-d column (Schleicher & Schuell). This procedure removed about 80% of the non-DNA reactive material (probably teichoic acid) which otherwise copurified with plasmid DNA during the isolation procedure. We found the recovery of plasmid DNA from the Elutip-d columns to be 70%.

CsCl density gradients. Solid CsCl (Ultra-Pure; Bethesda Research Laboratories) was added to a solution of plasmid DNA in 3.5 ml of buffer containing 0.01 M Tris, pH 7.5, and 0.001 M EDTA to give a refractive index of 1.4000 at room temperature. Centrifugation was carried out at 20°C in nitrocellulose tubes topped with mineral oil in a Ti50 rotor at 40,000 rpm for 60 h. Samples containing 1,000 cpm each of ^3H-labeled light and heavy plasmid DNAs were run in parallel to determine the positions of these markers in the gradient. ^3H-labeled light DNA was also added directly to the gradient containing ^{32}P-labeled DNA, for the same purpose. Three-drop fractions (about 30 μl) were collected from the gradients. A 200-μl portion of tRNA (50 μg/ml) was added as carrier to the radioactive samples, which were then precipitated with 400 μl of ethanol and centrifuged; the recovered pellets were dried and suspended in 20 μl of 4 mM NaCl–1 mM EDTA–10 mM Tris hydrochloride, pH 7.5. Aliquots were removed for trichloroacetic acid precipitation and determination of ^3H and ^{32}P label. Aliquots from the appropriate fractions were then electrophoresed on 0.8% agarose gels for 18 h at 30 V to separate plasmid DNA from chromosomal DNA. The slower migrating plasmid DNA bands from the ethidium bromide-stained gels were excised, and the agarose slices were melted by boiling in 2 ml of water. A 15-ml amount of Liquiscint (National Diagnostics) was added to each sample, and radioactivity was determined to measure the incorporation into plasmid DNA.

Determination of the specific activity of the deoxyribonucleotide pool in ^{32}P-labeled transformed cells. (i) Quantitation of radioactivity in dATP and TTP pools, using two-dimensional chromatography. The methods used to quantitate radioactivity are derived from Cashel et al. (7) and Randerath and Randerath (31). Half of each transformed, labeled, and washed cell suspension (2 ml) prepared as described above was treated with 160 μl of 2 M formic acid, held on ice for 15 min, and then clarified by centrifugation for 1 min at room temperature. Aliquots of the supernatant were applied to polyethyleneimine-cellulose F sheets (E. Merck AG). Identification of radioactive nucleotides was confirmed by their comigration with known concentrations of cold dATP and TTP added to extracts. Chromatographs were developed in the first dimension in 3.3 M ammonium formate, pH 7.0, plus 4.2% boric acid (for the resolution of dATP) and, for TTP, in "step formate," pH 3.4, with 0.5, 2, and 4 M sodium formate for 1 min, 2 min, and 2.5 h, respectively. Chromatograms were then air dried, washed in anhydrous methanol for 5 min, and developed in the second dimension in 0.85 M KH_2PO_4, pH 3.4. ^{32}P-labeled materials were located by autoradiography. Spots corresponding to dATP and TTP were identified under long-wavelength UV light, cut out, and suspended in 1 ml of 0.7 M $MgCl_2$–0.02 M Tris hydrochloride, pH 7.4. Optical density measurements at A_{260} were obtained from eluted material to verify recoveries. Radioactivities of the eluants were determined after the addition of 4 ml of Liquiscint (National Diagnostics) to 100-μl aliquots.

(ii) Determination of concentrations of dATP and TTP in extracts of transformed cells. The method described for determination of dATP and TTP concentrations is essentially that of Solter and Handschumacher (33) and Lindberg and Skoog (27) and is based on the ability of DNA polymerase, in the presence of poly(dA-dT) as primer template, to incorporate dATP and TTP into an acid-insoluble product. If either of the nucleoside triphosphates is limiting and the other is radioactively labeled and in excess, the incorporation of radioactivity is found to be directly proportional to the amount of the limiting nucleoside triphosphate. Washed and labeled cells (2 ml) were extracted twice with 6 volumes of 2 M $HClO_4$ at 4°C. The extracts were combined and immediately neutralized to pH 7.4 with KOH. The $KClO_4$ precipitate was removed by centrifugation and the extracts were assayed. A 1-ml amount of reaction mix contained 500 pmol of [^3H]TTP or [^3H]dATP (18 Ci/mmol), 5 nmol of poly(dA-dT) (Boehringer Mannheim), 50 mM $KHPO_4$ (pH 7.4), 6.7 mM $MgCl_2$, 1 mM mercaptoethanol, and 5 U of DNA polymerase I (Klenow fragment). A 0.2-ml portion of reaction mix was added to known amounts of cold dATP or TTP (0.4 to 2.8 pmol) and to aliquots of bacterial extracts. The samples were incubated at 37°C for 30 min, after which they were chilled, precipitated with trichloroacetic acid and dried; radioactivity was then determined. Standard curves were obtained for dATP and TTP, from which the concentrations of nucleotides in the unknown samples were determined.

Blotting and hybridization. Fractions collected from nonradioactive CsCl gradients were loaded onto BA85 nitrocellulose filters, using the Schleicher & Schuell 96-place microsample filtration manifold. The fractions (each about 30 μl) were brought to 95 μl by the addition of CsCl (55%, wt/wt) solution. A 5-μl amount of 2 N NaOH was then added, and the samples were heated at 80°C for 10 min, after which they were neutralized with 10 μl of 2 M Tris hydrochloride. Known concentrations of donor and recipient plasmid DNAs ranging from 1 to 250 ng were treated as above and blotted in parallel with fractions from the gradient. Two specific probes were prepared for hybridization. Total plasmid DNA was labeled by nick translation, using [α-^{32}P]-dATP and [α-^{32}P]dCTP to specific activities of

5×10^7 to 1×10^8 cpm/μg (32). The second probe was constructed with an *Sst*I-*Bam*HI fragment (270 base pairs) isolated from pBD235 DNA digests by electroelution. This fragment, which contains sequences from within the *hisH* gene and contained entirely within the Δ*hisH*1 deletion (Fig. 1), was cloned into M13mp18 as described by Messing and Vieira (28). The M13 probe was labeled by using a hybridization primer (New England BioLabs) as described by Hu and Messing (23) to yield specific activities of 3×10^8 to 1×10^9 cpm/μg. Filters were hybridized as described previously (24).

Autoradiograms were exposed at room temperature or at −70°C with DuPont Lightning-Plus intensifying screens for appropriate periods of time. Densitometer tracings of autoradiograms were made with a Joyce-Loebl densitometer. Areas under the peaks were measured by cutting out from the tracings and weighing. A standard curve was obtained from known concentrations of DNA, from which the concentrations of DNA in the gradient fractions were determined. Since the donor-specific probe was contained within the resident *hisH* deletion, transformation for the probe sequences was necessarily an all-or-nothing event. Thus, calculations from the standard curves yielded estimates proportional to the number of plasmid DNA molecules that had been transformed.

RESULTS

To distinguish between the copy choice and breakage-reunion mechanisms, the various moieties of the recombinant plasmids resulting from PMRT were measured. These included integrated donor material, newly synthesized material associated with recombinant DNA, and the resident plasmid DNA contribution.

Estimation of donor and total plasmid DNA in transformed cells. Donor DNA was identified by use of heavy (^2H,^{15}N-labeled) plasmid DNA in the transformation assay, which allowed detection by density displacement in CsCl gradients of DNA molecules containing atoms of donor origin. If donor DNA was incorporated into recombinant DNA, the latter would contain a heavy donor moiety associated with the light DNA (either newly synthesized or preexisting) and would display a density between light (L/L) and hybrid (H/L), assuming that only single donor strands are integrated. The use of donor-specific probes permitted the identification of DNA carrying donor genetic information in fractions from CsCl gradients.

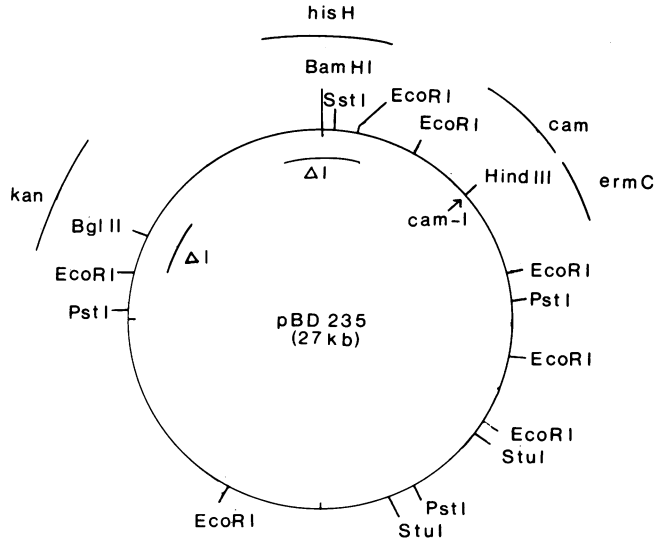

FIG. 1. Physical map of pBD235. Essential restriction enzyme cleavage sites and the locations of the *kan*, *cam*, *ermC*, and *hisH* determinants are shown. The *cam-1* mutation was constructed by the introduction of an 8-base *Hin*dIII linker into the *Stu*I site that occurs in the wild-type *cam* gene (34). The locations of two mutations in the resident plasmid (pBD237) are also shown (34). The 1-kb *kan*Δ*1* mutation was introduced by Bal31 digestion. The *hisH*Δ*1* mutation is a spontaneous 2.5-kb deletion.

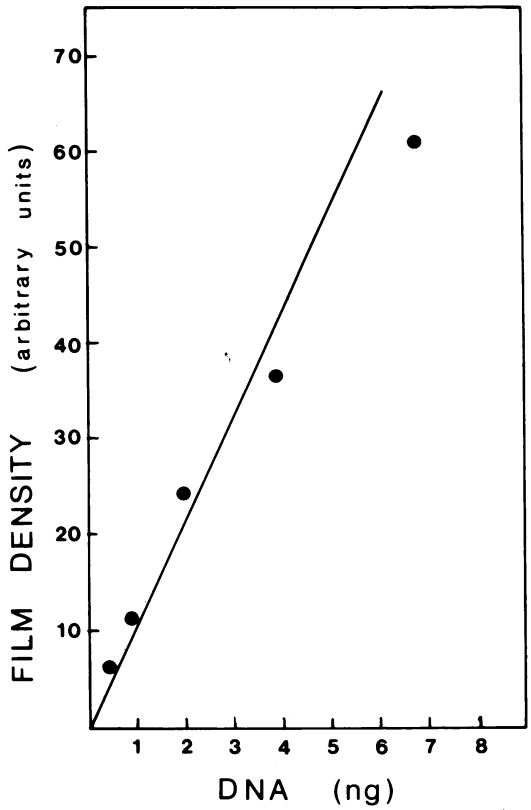

FIG. 2. Standard curve relating concentrations of plasmid DNA with film density determined by autoradiography. Known concentrations of pBD235 DNA were blotted onto a nitrocellulose membrane and hybridized with a radioactive plasmid DNA probe. The filters were autoradiographed, and film densities were determined from tracings as described in Materials and Methods.

Linear plasmid pBD235 DNA (*kan*$^+$ *cat-1 hisH*$^+$ *ermC*$^+$) was used to transform cells carrying the resident plasmid pBD237 (Δ*kan-1 cat*$^+$ Δ*hisH1 ermC*$^+$). The Δ*hisH1* mutation is a deletion of 2.5 kbp. The entire donor plasmid consists of 27 kbp, a length within the optimal range for PMRT (34). Transformation was terminated after 20 min by the addition of DNase, and the DNA was extracted and analyzed on CsCl gradients. Fractions were collected and blotted on nitrocellulose membranes together with standards containing known concentrations of donor and recipient DNA. The filters were hybridized with M13 DNA into which a donor-specific fragment had been inserted. This fragment consisted of a sequence from within the *his* gene not present in the resident plasmid. After decay, the same filters were hybridized to nick-translated resident DNA to permit the estimation of total plasmid DNA in each fraction. Standard curves (Fig. 2) were obtained from densitometer tracings of the resulting autoradiograms. From this, the contents of

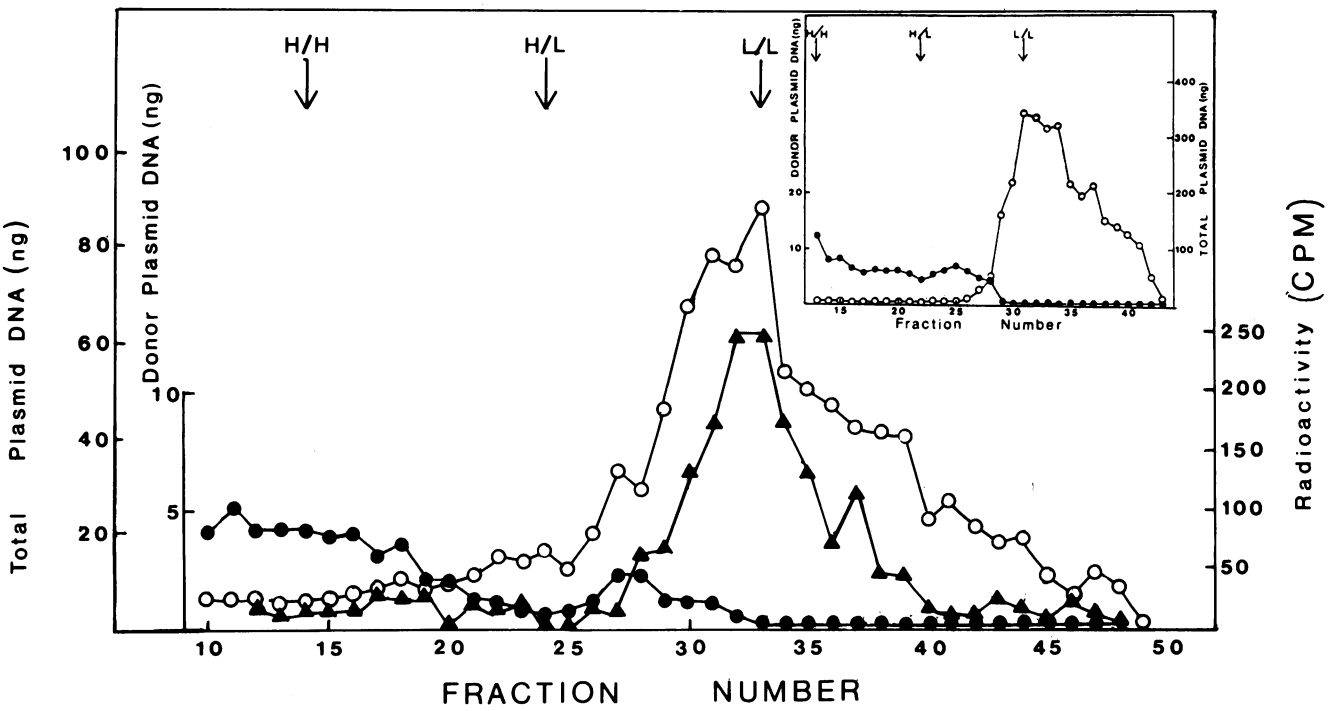

FIG. 3. Distribution of donor (●), total (○), and newly synthesized (▲) plasmid DNA in a CsCl gradient after transformation with $^{2}H,^{15}N$-labeled DNA. Linear $^{2}H,^{15}N$-labeled pBD235 DNA was used to transform cells of BD630 carrying pBD237 in the presence of $^{32}P_i$. Plasmid DNA isolated from the transformed culture was subjected to CsCl gradient centrifugation as described in Materials and Methods. The positions of heavy (H/H), hybrid (H/L), and light (L/L) plasmid DNA are indicated by arrows. The inset shows the distribution of donor and total plasmid DNA in an independent experiment.

donor and total plasmid DNA in each fraction were determined and plotted as shown in Fig. 3. A major peak of total plasmid DNA was located at the position of the L/L marker. This contained 912 ng in the experiment shown. Donor-specific DNA appeared to resolve into two components. The first was a broad band overlapping the H/H marker. The second was a peak (found in repeated experiments) distinct from the broad band and located between H/L and the L/L marker. No peak with donor specificity was observed to be centered at the L/L position. The donor DNA in the second peak contained 1.4% of the total plasmid DNA. This agreed reasonably with the transforming frequency (1.7%) for the Δ*his-1* marker. Similar results were obtained in several experiments. A second, similar experiment is illustrated in the inset in Fig. 3. We consider the second peak to consist of recombinant plasmid DNA and the first component to consist of low-molecular-weight donor DNA, probably single stranded. Such small single-strand fragments of donor origin are known to be formed during both CT (14) and plasmid transformation (10). Another experiment (not shown) was performed, in which light donor DNA was used. The intracellular donor material hybridized at the L/L position, with some material hybridizing at a position shifted toward the heavy side. No donor DNA was detected at a position shifted toward the light side of the L/L peak. This strengthens our interpretation of the nature of the intermediate density peak noted above.

Estimation of newly synthesized DNA in transformed lysates. Cells from the same experiment were transformed as described previously but in the presence of ^{32}P. The specific activity of the internal pool was estimated from samples removed after 5, 10, and 20 min (see Materials and Methods). Results are presented in Fig. 4. The specific activities increase from time zero and reach a plateau at about 10 min.

Plasmid DNA, isolated from lysates following transformation in the presence of ^{32}P, was resolved on CsCl gradients. As described in Materials and Methods, it was important to separate the plasmid DNA from contaminating radioactive material, presumably teichoic acid, by the use of Elutip-d

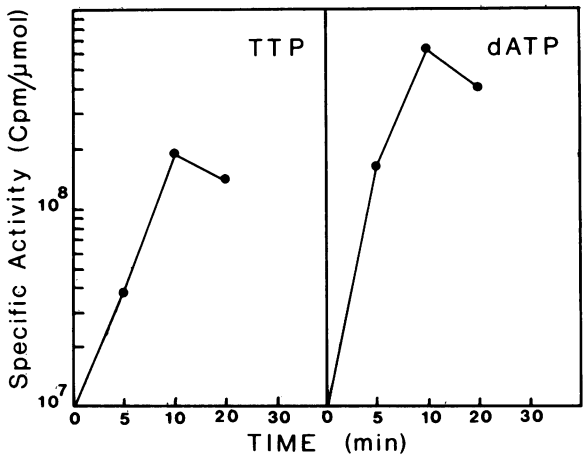

FIG. 4. Specific activities of the TTP and dATP pools in a ^{32}P-labeled transformed culture. The concentrations of TTP and dATP in the internal pool were determined by using a DNA polymerase assay, as described in Materials and Methods. The radioactivity present in the dATP and TTP pools was measured by two-dimensional thin-layer chromatography.

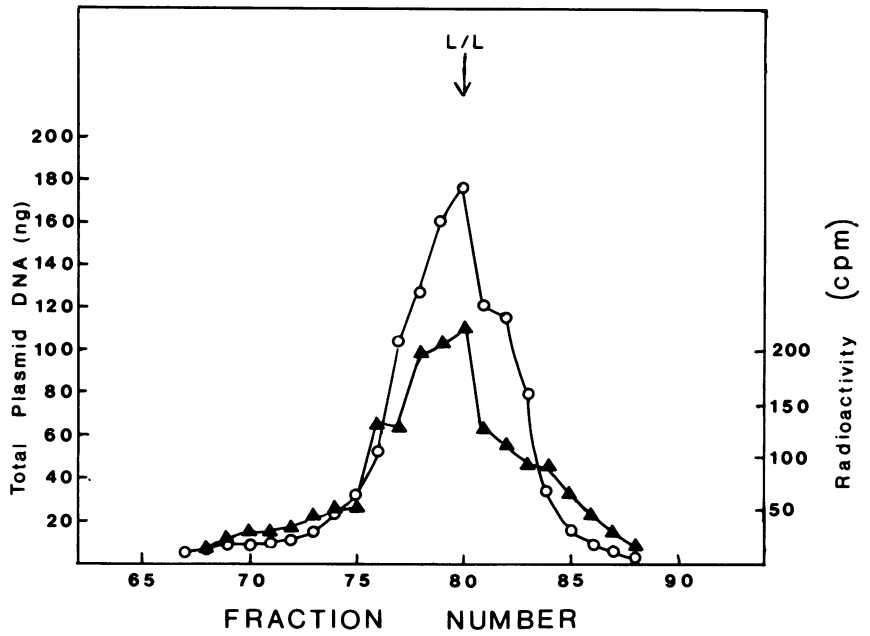

FIG. 5. Distribution of total (O) and newly synthesized (▲) plasmid DNA in a CsCl gradient after incubation without transformation. Plasmid DNA was isolated from an untransformed BD630 culture after incubation in the presence of $^{32}P_i$.

columns. To isolate plasmid DNA free of chromosomal DNA, appropriate fractions were precipitated and run on 0.8% agarose gels, which were stained and photographed. Gel slices containing plasmid bands were excised and the radioactivity was determined. Figure 3 shows a profile of radioactive incorporation in newly synthesized DNA. Profiles of total and newly synthesized DNA from similarly treated untransformed competent cells are also shown for purposes of comparison (Fig. 5). In both the transformed and untransformed samples, the new newly synthesized material bands at the L/L position, coincident with the peak of total DNA. The relative extends of DNA synthesis are similar in the two samples.

DISCUSSION

We have used biochemical techniques to investigate the formation of recombinant plasmid DNA molecules during PMRT in competent cells of *B. subtilis*. Previous data had demonstrated that, with regard to a variety of genetic and biochemical parameters, PMRT was similar to the transformation of chromosomal markers (8, 34). The present study seeks to determine whether PMRT, like CT, proceeds by a breakage-reunion mechanism, in which donor DNA is physically joined to old resident DNA, or exclusively by a copy choice mechanism, in which all of either the donor or resident genetic information on a recombinant strand is copied by DNA synthesis.

The use of heavy-labeled donor DNA demonstrates that recombinant DNA contains donor atoms. The amount of DNA in the density-displaced fraction comprised 1.4% of the total plasmid DNA extracted from transformed cells. In several experiments we have observed that the plasmid DNA recovered from the CsCl gradients migrated in agarose gels with the mobility expected for unit-length molecules. It appears, then, that about 1.4% of the plasmid molecules in transformed cells contained segments of contiguous donor DNA of sufficient length to cause a perceptible density displacement. The donor-specific probe used in making these determinations is derived from within the *hisH* gene, and the transformation frequency for the resident plasmid *hisH* deletion was 1.7%. Since these two estimates correspond reasonably well, we conclude that at least a majority of transformants can be accounted for by the joining of heavy donor DNA to light segments derived either from old resident plasmids or by synthesis of new material. This rules out a class of copy choice models, in which genetic information is copied into a recombinant molecule from a donor template.

The extent of the density shift exhibited by the recombinant molecules can be used to estimate the average size of the integrated donor moiety. This has been measured by physical means in the case of *B. subtilis* CT as 8.5 kilobases (kb) in one report (13) and as 10 kb in another (18). Furthermore, both of these studies revealed that integration events were clustered, with an average of two to four donor fragments inserting near one another. The modal density shift in Fig. 3 is 0.29 as a fraction of the distance between L/L and H/H markers. Since the recombinant plasmid consists of 54 kb (27 kbp), this represents an average donor insertion of 16 kb. It is likely that donor single strands are integrated. We have shown that, as with CT, plasmid DNA is taken up in single-stranded form and no double-stranded intracellular DNA is detectable (unpublished data). If so, our results may be interpreted as implying two clustered insertions of an average of 8 kb each into the 54-kb recombinant plasmid. The latter interpretation agrees well with those from physical studies of chromosomal transformation described above. The clustering phenomenon has been explained (12, 13, 18) by the fragmentation of high-molecular-weight transforming DNA on the competent cell surface (14) together with the high efficiency of integration following uptake. Fragments derived from the same donor molecule will therefore integrate near one another, resulting in clustering. In the present experiments the donor pBD235 DNA was cut with *Stu*I, yielding two fragments with respective

lengths of about 3 and 24 kbp. With the latter donor fragment, further fragmentation on the competent cell surface would be expected to occur an average of once per molecule at a random position, yielding a population of molecules with a number average molecular length of 12 kbp. This prediction is based on model studies of the fragmentation of the similarly sized coliphage T7 DNA (11). The smaller (3-kbp) fragment may be disregarded since the steep size dependence of PMRT would exclude it from contributing significantly to the formation of recombinant molecules (34). Evidence from two different experimental approaches has suggested that an "excluded length" of about 1 kb is removed from the ends of *B. subtilis* transforming DNA prior to integration (8, 22). Thus, two segments, each containing about 10 kbp, would be produced by surface fragmentation of a plasmid molecule, yielding intracellular single-stranded fragments of 10 kb. Intracellular donor single strands averaging 8 to 9 kb have been detected during CT of *B. subtilis* (15). A clustering of two single-strand insertion events, each averaging 8 kb, is therefore reasonable and consistent with the current data for CT.

It remains to estimate the contribution of newly synthesized DNA to the recombinant molecules. Figure 2 shows that the specific activities of the TTP and dATP pools vary during the course of the experiment, rising rapidly to a plateau at 10 min. The data in Fig. 3 show the cumulative incorporation of $^{32}P_i$ into DNA at 20 min. The uptake of single-strand donor DNA begins after a lag of 2 min (9; unpublished data). Thus, the synthesis of new material shown in Fig. 3 must have occurred between min 2 and 20. If we use the average plateau minute value for the specific activity of the pool (3.8×10^8 cpm/μmol), the calculated value for the amount of newly synthesized DNA in the fractions (24 to 33) corresponding to recombinant plasmid DNA is 618 pg or 5.4% of the recombinant DNA in these fractions. If all of this synthesis occurred in recombinant DNA, this would correspond to 2.9 kb per recombinant molecule. If we use instead the more conservative 5-min value for specific activity (10^8 cpm/μmol), the calculated value would be 2,349 pg of newly synthesized DNA, corresponding to 20.5% of the recombinant plasmid DNA, or 11.1 kb. However, both of these are gross overestimates. Inspection of Fig. 3 reveals that the profile of $^{32}P_i$ incorporation coincides with that of total DNA, not the recombinant DNA peak. It is thus not reasonable to assume that all newly synthesized DNA in fractions 24 to 33 is in recombinant DNA. Furthermore, in Fig. 5 it can be seen that, in an identically treated sample from untransformed competent cells, a similar peak of newly synthesized DNA also coincides with the total DNA band. In a separate experiment (not shown), in which light donor DNA was used, a peak of radioactivity was again observed that coincided with that of total plasmid DNA, and no displaced band of donor specificity was observed. In these three cases similar extents of total DNA synthesis were obtained as a percentage of total plasmid DNA: 0.16% (heavy donor), 0.16% (light donor), and 0.12% (no donor). The estimated DNA synthesis in the fractions corresponding to 24 to 33 was 0.13% of the total plasmid DNA in those fractions for the sample transformed with heavy DNA and 0.24 and 0.13% in the equivalent fractions for the other two samples. It is clear that the bulk of newly synthesized plasmid DNA is associated with the nonrecombinant molecules and that the estimates given above for the extent of synthesis associated with recombination are excessive. A different approximation to the extent of DNA synthesis in the recombinant DNA can be made by correcting the synthesis in fractions 24 to 33 by the ratio of recombinant/total DNA in those fractions. This assumes that synthesis is randomly distributed with respect to recombinant and nonrecombinant DNA. The fraction of total DNA present in recombinant molecules within fractions 24 to 33 is 2.4×10^{-2}. If we use this value to correct the estimate of newly synthesized DNA in recombinant molecules based on the conservative 5-min pool specific activity, we arrive at the conclusion that about 266 bases of newly synthesized DNA are present in the average recombinant molecule. The incorporation measured in these experiments represents repair and not DNA replication, because of the presence of a high concentration of 6-(*p*-hydroxyphenylazo)-uracil.

We have estimated (see above) that about 16 kb of donor DNA is incorporated per recombinant molecule. This leaves 38 kb to be accounted for. Even if our estimate for the extent of DNA synthesis associated with recombination is too low by a factor of 100, it is evident that at least some old recipient plasmid DNA must be present in the average recombinant molecule. We can consider two models, both consistent with the presence of heavy donor DNA and light resident DNA in the recombinant plasmid. In one, a heavy donor strand pairs with a recipient strand and is completed by repair replication, using the recipient strand as a template. This would require an average of 11 kb of synthesis if the entire 16 kb of donor DNA is present on one strand. This model seems unlikely, given the estimates described above. In a second model, the donor single strand is inserted into a recipient strand and covalently joined to old recipient DNA, possibly with some repair synthesis needed to fill in gaps. The latter model appears to be more reasonable, given the numerical estimates of repair synthesis given above, although the first possibility cannot be rigorously excluded. In any event, the recombinant plasmid contains preexisting donor and recipient DNA, possibly but not necessarily in addition to some newly synthesized material, and is therefore the product of a breakage-reunion type of mechanism.

Since the amount of density-shifted DNA formed is consistent with the transformation frequency, and the latter is unaffected by the presence of 6-(*p*-hydroxyphenylazo)-uracil (not shown), we conclude that most, if not all, PMRT proceeds via a mechanism that does not require DNA replication. In the latter respect, PMRT also resembles CT (16, 26), and models involving recombination between the progeny of donor and recipient plasmids present at the time of transformation are ruled out.

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