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Construction of a Colicin E1-R Factor Composite Plasmid In Vitro: Means for Amplification of Deoxyribonucleic Acid

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A composite plasmid has been constructed in vitro from colicin E1 factor (mass of 4.2 megadaltons [Md]) and nontransmissible resistance factor RSF 1010 (mass, 5.5 Md) deoxyribonucleic acids (DNAs) by the sequential action of *Escherichia coli* endonuclease RI (Eco RI) and T4 phage DNA ligase on the covalently closed circular forms of the constituents. The composite plasmid was selected and amplified in vivo by sequential transformation of *E. coli* C600 with the ligated mixture and selection of transformants in medium containing streptomycin plus colicin E1, followed by amplification in the presence of chloramphenicol and purification of the extracted plasmid by dye-buoyant density gradient centrifugation in ethidium bromide-cesium chloride solution. Treatment of the composite plasmid with Eco RI yielded two fragments with mobilities corresponding to the linear forms of the parental plasmids, whereas *Serratia marscesens* endonuclease R (Sma R), which introduces a single scission in the colicin E1 factor but not in RSF 1010, converted the composite plasmid to a single linear molecule (mass, 9.7 Md). Sequential degradation of colicin E1 factor with Sma R and Eco RI produced two fragments with masses of 3.5 and 0.7 Md; sequential degradation of RSF 1010 produced only one fragment (due to the cleavage with Eco RI), and sequential degradation of the composite plasmid produced the expected three fragments—an RSF 1010 Eco RI linear and the two expected products from the colicin E1 factor moiety. The composite plasmid conferred on the host cell resistance to streptomycin, sulfonamides, and colicin E1, but colicin E1 itself was not synthesized. In contrast, colicin E1 was synthesized by cells containing simultaneously both colicin E1 factor and RSF 1010 as separate entities. In the presence of chloramphenicol, the composite plasmid continued to replicate for 6 h, whereas replication of RSF 1010 and chromosomal DNA stopped within 2 h. Continued replication in the presence of chloramphenicol suggests that the replicator of the colicin E1 factor is functional in the composite plasmid.

The possibility of constructing composite plasmids has recently been reported by Cohen et al. (3). In these studies, based on the demonstration of Mertz and Davis (9) that Eco RI cleaves deoxyribonucleic acid (DNA), leaving cohesive termini capable of covalent closure with DNA ligase, composite plasmids were prepared by attachment of various plasmid DNAs to a tetracycline resistance plasmid used as replicator.

In the present studies, construction in vitro of a new composite plasmid from colicin E1 factor and RSF 1010 is reported together with a description of its biological properties as well as patterns of enzymatic cleavage using restriction endonucleases. In the composite plasmid, desired biological functions from the parental plasmids are combined; these include continued replication in the presence of chloramphenicol coupled with the ease of selection for streptomycin resistance. These features make the composite factor potentially useful in connection with further clonal selection and amplification of DNA.

MATERIALS AND METHODS

Bacterial strains. The following strains were used. *Escherichia coli* JC411 carrying colicin E1 factor was obtained from J. Konisky. *E. coli* W1485 carrying R factor RSF 1010 was obtained from S. Falkow; this strain is resistant to streptomycin and sulfonamides. *E. coli* C600 used as a recipient in transformation experiments was obtained from M. Nomura.

Media. Enriched medium contained (in grams per liter): peptone, 5; yeast extract, 5; K_2HPO_4, 1; and glucose, 2. M-9 medium plus Casamino Acids contained (in grams per liter): NH_4Cl, 1; Na_2HPO_4, 6;

KH_2PO_4, 3; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.015; glucose, 2; and Casamino Acids, 20. The components were mixed and sterilized by passage through a membrane filter (HAWP 02500; Millipore Corp.).

Plasmids. Colicin E1 factor was prepared by an adaptation of the method of Clewell (2) as follows. JC411 was grown overnight in enriched medium and diluted 10-fold into M-9 supplemented with 2% Casamino Acids and 0.2% glucose. The culture was permitted to grow for an additional 2 h with vigorous agitation on a rotatory shaker, during which time the original inoculum increased two- to fourfold. Chloramphenicol was added to a final concentration of 170 μg/ml (by 200-fold dilution of a stock solution containing 34 mg/ml in ethanol), and incubation continued for an additional 8 h. Cells were harvested and lysates were prepared by the method of Guerry et al. (5). For preparation of RSF 1010, cells of *E. coli* W1485 carrying this plasmid were grown to late log phase in enriched medium, harvested, and lysed as for preparation of colicin E1 factor. Details of the lysis step and centrifugation in cesium chloride-ethidium bromide solution are presented below under stage 4.

Enzymes. For preparing restriction enzymes, the following solutions were used: 10 × EXB — KPO_4, (pH 7.0, 100 mM), disodium ethylenediaminetetraacetate (Na_2EDTA) (10 mM), and 2-mercaptoethanol (70 mM); EXB — 10-fold dilution of 10 × EXB; EXBT — EXB containing 0.15% Triton X-100. Eco RI purification was based on a combination of methods devised by Greene et al. (P. J. Greene, M. Betlach, H. M. Goodman, and H. Boyer, *In* R. B. Wickner (ed.), *Methods in Molecular Biology*, in press) and J. E. Davies (personal communication) as follows: *E. coli* strain RY-13 (endonuclease I-deficient) grown to stationary phase in enriched medium was used as enzyme source. Ten grams of cell paste was suspended and washed successively with tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.4 (10 mM), and NaCl (30 mM) (twice), suspended in 50 ml of Tris-hydrochloride, pH 7.4 (33 mM), Na_2EDTA (3 mM), and sucrose (20% [wt/vol]) and incubated for 10 min at 25 C. Cells were collected by centrifugation (12,000 × *g*; 15 min, 4 C) and suspended in 50 ml of 0.5 mM $MgCl_2$. The supernatant (50 ml) was collected by centrifugation as above. To the supernatant were added 0.6 ml of Triton X-100, 15% in water, and 6 ml of 10 × EXB. The sample was applied to a column of diethylaminoethyl-cellulose (2.5-cm diameter by 20 cm) equilibrated with EXBT, and the enzyme was eluted with a gradient of 0.0 to 0.3 M NaCl all in EXBT (total volume, 500 ml). Enzymatic activity emerging at approximately 0.2 M NaCl was tested by using 5-μliter samples of column fractions according to the method described below. The active fractions were pooled, diluted fivefold with EXBT, and applied to column of phosphocellulose (P-11, Whatman) (2.5-cm diameter by 20 cm) equilibrated with EXBT, and the enzyme was eluted with a gradient of 0.05 to 0.60 M NaCl in EXBT (total volume, 500 ml). Active fractions emerging at approximately 0.4 M NaCl were located as above and dialyzed against EXBT containing NaCl (0.3 M) and glycerol (50%, vol/vol). The enzyme, concentrated approximately threefold by this procedure, showed no detectable loss of activity during storage at −20 C for 6 months. Enzyme activity was roughly quantitated by noting complete digestion of λ phage DNA substrate if a 10-fold dilution of the enzyme into the reaction mixture was used, whereas 100-fold dilution of enzyme produced only a partial digest.

Because concentrations of phosphate in excess of 0.15 mM were found to inhibit transformation (stage 3), the Eco RI preparation was dialyzed against Tris-hydrochloride, pH 7.4 (10 mM); Na_2EDTA (1 mM); NaCl (300 mM); 2-mercaptoethanol (7 mM); and Triton X-100 (0.15%) before use in order to remove phosphate present in the storage buffer. Under these conditions, the phosphate-free enzyme was stable for at least 1 month during storage at 4 C.

Sma R purification was according to the method of Mulder (manuscript in preparation) as follows. *S. marscesens* strain SB grown to stationary phase in enriched medium was used as enzyme source. Twenty grams of cell paste, suspended in 80 ml of EXB, was disrupted by passage through a French press. Ribosomes were removed by ultracentrifugation, and residual nucleic acids were removed by addition of 20 ml of freshly prepared 5% streptomycin sulfate solution (in water). The streptomycin supernatant was further fractionated with ammonium sulfate (50 to 80%), and the pellet, taken up in and dialyzed against EXB, was applied to a diethylaminoethyl-cellulose column (2.5-cm diameter by 20 cm) and eluted with a gradient of 0.0 to 0.6 M NaCl in EXB (total volume, 500 ml). The active fractions, emerging between 0.4 and 0.6 M NaCl, were dialyzed against 0.05 M NaCl in EXB and applied to a phosphocellulose column as above (P-11, Whatman), and the enzyme was eluted with a gradient of 0.05 M to 0.6 M NaCl in EXB (total volume, 500 ml). The activity emerged between 0.5 and 0.6 M NaCl. Active fractions were dialyzed against EXB containing NaCl (0.3 M) and glycerol (50%, vol/vol). The enzyme, concentrated approximately threefold by this procedure, showed no detectable loss of activity during storage at −20 C for 6 months.

DNA ligase. A commercial product prepared from phage T4-infected cells of *E. coli* B was purchased from the Miles Co., Kankakee, Ill. (control no. 39-6-625). The preparation, nominally purified through the phosphocellulose step (fraction VI) of Weiss et al. (11) was found to be active and sufficiently free of endo- or exonucleases to be suitable for these studies as shown by the fluorometric assay for DNA ligase described below.

Digestion of DNA samples with endonuclease. For digestion with Eco RI, the incubation mixture (total volume, 50 μliters) contained: Tris-hydrochloride, pH 7.4 (90 mM); $MgCl_2$ (10 mM); plasmid DNA (1 μg); and Eco RI preparation (5 μliters). The mixture was incubated at 37 C for 15 min, and the reaction was terminated by addition of 35 μliters of BJ solution (Na_2EDTA, 50 mM; sucrose, 30% [wt/wt]; bromophenol blue, 0.005%). For digestion with Sma R, the incubation mixture (total volume, 50 μliters) contained: Tris-hydrochloride, pH 9.0 (15 mM);

$MgCl_2$ (6 mM); KCl (15 mM); plasmid DNA (1 μg); and Sma R (5 μliters). The mixture was incubated at 37 C for 30 min, and the reaction was terminated as above.

Qualitative fluorimetric assay of ligase activity. Ligase activity was checked by incubating 1 μg of linear RSF 1010 or colicin E1 factor prepared as described in stage 1 below with ligase as described in stage 2. From the final reaction mixture, 50 μliters was subject to agarose-ethidium bromide electrophoresis as described below after addition of 35 μliters of BJ solution in order to stop the reaction. A "typical" reaction (done four times), in which 1 μg of linear RSF 1010 and 1 μliter of T4 ligase (nominally 0.1 nanomolar unit) were used, produced approximately 70% conversion of the linear to equal amounts of relaxed circular and supercoiled forms. In addition, trace amounts of new bands tentatively identified as dimeric and trimeric linear forms could also be visualized in the gel. Moreover, superhelical RSF 1010 incubated with ligase under conditions described in stage 2 showed almost no (less than 10%) conversion to the relaxed circular form and no detectable conversion (less than 10%) to the linear form.

Agarose-ethidium bromide gel electrophoresis. The method of Sharp et al. (10) was used for electrophoretic analysis of DNA samples. Standard conditions for electrophoresis were the following. Samples (50 μliters) mixed with 35 μliters of BJ solution were applied to agarose gels prepared as described. Electrophoresis buffer was prepared by dilution of a 10-times-concentrated stock solution (10 × electrophoresis buffer) containing Tris base (0.4 M), NaAc (0.2 M), glacial acetic acid (15 ml/liter), Na_2EDTA (0.01 M), and ethidium bromide (0.5 mg/liter), pH 8.0. Electrophoresis was performed by applying 50 V across the agarose gels (10 cm in length) long enough for the bromophenol blue tracking dye to reach the end of the gel, generally 3 to 4 h.

For direct electrophoretic analysis of controlled lysates, 20 μliters of ethidium bromide (0.1%) was also added directly to the sample in order to ensure the availability of sufficient ethidium bromide for intercalation into the DNA. If the additional ethidium bromide was omitted, anionic material, presumably sodium dodecyl sulfate (SDS), ran ahead of the tracking dye and preferentially adsorbed ethidium bromide added to the gel; this precluded direct visualization of DNA during fractionation and necessitated an additional staining step upon completion of the run. Direct visualization of the sample during fractionation without removal from the glass tube was possible with the use of a long-wave ultraviolet source (model B-100 A, U.V. Products, San Gabriel, Calif.). Photographic records were prepared with a Polaroid MP-3 camera and type 57 film (ASA 3000) by illuminating gels with the ultraviolet source. Other conditions for photography were 10- to 20-s exposure at f 4.7 with filters number 25A red and number 8 yellow (Tiffen Corp., Roslyn Heights, N.Y.).

Preparation of the composite plasmid. Stage 1: Preparation of substrates. RSF 1010 and colicin E1 factor were digested with Eco RI in order to convert them to the respective linear forms with cohesive ends. The reaction mixture contained Tris-hydrochloride, pH 7.4 (90 mM); $MgCl_2$ (10 mM); plasmid (1 μg each of RSF 1010 and E1 factor); and Eco RI (5 μliters) in a final volume of 50 μliters. The mixture was incubated at 37 C for 1 h and the reaction was terminated by heating to 65 C for 5 min. Completeness of digestion was determined by electrophoresis of a 25-μliter sample in agarose as described above.

Stage 2: Ligation. The ligase reaction was performed in a final volume of 100 μliters. The sample described in step 1 was placed on ice and the following were added: 50 mM MgCl (10 μliters); 0.1 M dithioerythritol (10 μliters); 0.5 mM adenosine triphosphate (10 μliters); water (20 μliters); and T4 ligase (100 nanomolar U/ml; 1 μliter). The reaction mixture was incubated on ice for an additional 18 h, and 3 μliters of 1 M $CaCl_2$ was added immediately before addition of the ligated reaction mix to competent cells for transformation; final $CaCl_2$ concentration was 30 mM.

Stage 3: Transformation, selection, and amplification. A culture of *E. coli* C600 was treated with $CaCl_2$ solution according to the method of Mandel and Higa (8) in order to render it competent for tranformation. The competent cell suspension (200 μliters) was kept on ice and the incubated ligase mixture (100 μliters; stage 2) was added with a cold pipette. The transformation mixture was incubated for 30 min on ice, further incubated at 37 C for 20 min, and centrifuged to remove $CaCl_2$ solution, after which the cells were resuspended in 10 ml of enriched medium and incubated at 37 C with vigorous agitation. The culture was allowed to grow to maximal turbidity (about 10^9 cells/ml, requiring about 3 h), and 0.1 ml was inoculated into 10 ml of enriched medium containing streptomycin (10 μg/ml) and colicin E1 (10 U/cell) for selection of transformants. Colicin E1 was prepared according to the method of Foulds and Barrett (4). After the culture grown under these selective conditions reached maximal turbidity, requiring approximately 12 h of incubation with vigorous agitation, it was added to 90 ml of prewarmed (37 C) M-9 medium plus 2% Casamino Acids and 0.2% glucose and incubated for an additional 2 h at 37 C with vigorous agitation, at which time the culture, having increased in turbidity approximately fourfold, received chloramphenicol (34 mg/ml) dissolved in ethanol (500 μliters; final concentration in the culture medium, 170 μg/ml) in order to differentially amplify plasmids utilizing the colicin E1 factor replicator. The culture was incubated for an additional 6 h at 37 C with vigorous agitation and then chilled. Cells were harvested by centrifugation, and plasmids were extracted by an adaptation of the method described by Guerry et al. (5) as follows.

Stage 4: Plasmid extraction and purification. The pellet from 100 ml of culture in the previous step was suspended in 1.0 ml of Tris-sucrose (Tris-hydrochloride, pH 8.0 [50 mM]; sucrose [25% wt/vol]); 0.2 ml of lysozyme (5 mg/ml in water) was added, and the mixture was incubated on ice for 5 min. Next, 0.4 ml of Na_2EDTA (0.25 M, pH 8.0) was added and the mixture was incubated on ice for an additional 5 min. NaCl (0.5 ml, 5 M) and 10% SDS (0.2 ml) were added and mixed in succession. Special care was taken to

disperse the SDS as rapidly as possible by use of a mechanical Vortex mixer since the mixture became extremely viscous upon contact with the SDS solution. The mixture was incubated on ice till the next working day, generally 10 to 12 h, at which time it was spun (30,000 × *g*, 30 min, 4 C). A clear, nonviscous supernatant fluid was carefully aspirated (total volume, 2.5 ml) and made up to a final volume of 5 ml with 50 mM Na_2EDTA. Solid CsCl (4.7 g) and ethidium bromide (1.8 mg) were added, and the final density was adjusted to 1.57 g/ml. The sample was spun at 15 C and 40,000 rpm for 40 h in a Spinco 65 rotor. The presence of superhelical DNA was ascertained by viewing the tube under ultraviolet illumination as described by Clewell (2), and 0.3-ml fractions were collected with the aid of a peristaltic pump as above. The combined fractions containing superhelical DNA (total volume, 1.0 ml) were extracted three times with 2 ml of isoamyl alcohol in order to remove ethidium bromide, and CsCl was removed by dialysis against 0.1 × SSC (0.15 NaCl + 0.015 sodium citrate) for 6 h.

Stage 5: Enrichment for composite plasmids by preparative agarose gel electrophoresis. Superhelical DNA from stage 4 (0.40 ml) was mixed with 0.25 ml of BJ solution, and 80-μliter samples of this mixture, layered on agarose gels (0.7%, 6-mm diameter), were subject to electrophoresis for 4 h at 50 V. Under ultraviolet illumination the gels were found to contain several slowly migrating bands in addition to faster-moving bands whose mobilities coincided with those of the conformers of the parental DNAs. Segments from each gel close to the origin containing DNA bands with mobilities slower than relaxed circles of RSF 1010 were excised with a razor blade. The combined gel segments were collected in the barrel of a sterile 5-ml plastic syringe and extruded through a size 20 hypodermic needle into 2 ml of cold Tris-hydrochloride, pH 8.0 (10 mM); NaCl (0.3 M); and Na_2EDTA (10 mM). The suspension was stored on ice overnight, frozen at −50 C for 3 h in order to disrupt the gel matrix, and thawed, and gel particles were removed by centrifugation at 30,000 × *g* for 30 min at 4 C. The supernatant solution was filtered through a membrane filter (HAWP-02500; Millipore Corp.) in order to remove fine particles of agarose, extracted three times with isoamyl alcohol to remove ethidium bromide, and dialyzed against 0.1 × SSC + Na_2EDTA (1 mM).

Stage 6: Second transformation. Competent cells of *E. coli* C600 prepared as above (200 μliters) and composite plasmid from stage 5 (100 μliters in 30 mM $CaCl_2$) were mixed and incubated as in stage 3. The transformation mixture (300 μliters) was pipetted into 10 ml of enriched medium, and the culture was allowed to grow to maximal turbidity, requiring about 3 h, followed by selection with streptomycin and colicin E1 as above. A loopful of fully grown culture was spread onto solid medium (enriched broth containing 2% agar) supplemented with streptomycin sulfate (10 μg/ml) for isolation of individual clones.

Stage 7: Screening for new plasmids. Sixteen colonies from stage 6 were picked and grown overnight in 1 ml of enriched medium. A controlled lysate was prepared from each sample after successive addition of M-9 medium plus Casamino Acids and chloramphenicol at 1/10 the scale described for stages 3 and 4 above. From each lysate, 25 μliters was subjected to electrophoresis by the agarose-ethidium bromide technique of Sharp et al. (10). Most of the gels showed the presence of discrete bands that could not be accounted for by the presence of any of the conformers of RSF 1010 or colicin E1 factor. One of the samples was chosen for further study.

Stage 8: Preparation of the composite plasmid. Cells from the clone selected for further study were repurified. A single clone was grown in enriched medium, which was in turn diluted 10-fold into M-9 medium containing 2% Casamino Acids and 0.2% glucose. After treatment with chloramphenicol as in stage 3, plasmids were extracted and purified in the same proportions given in stage 4. Superhelical DNA was finally purified by two successive bandings in cesium chloride-ethidium bromide solution.

Molecular weight measurements. The molecular weight of the composite plasmid was determined by electrophoresis in 0.7% agarose by comparison with a set of standard fragments generated with Eco RI by using DNA from phage λ (cb2) as substrate. The molecular weights reported by Allet et al. (1) for these fragments were used in the calibration.

RESULTS

A schematic structural outline of the composite fractor including the approximate relative positions of the endonucleolytic cleavage sites of Eco RI and Sma R enzymes is presented in Fig. 1.

Degradation of the composite factor with restriction endonucleases. Analysis of the fragments obtained by digestion of the parental plasmids RSF 1010 and colicin E1 factor with restriction endonucleases is shown in Fig. 2. In these separations, three different forms of each plasmid could be distinguished. The relative mobilities of these isomeric forms, starting from the origin were: relaxed circle, linear, and supercoil. The undigested colicin E1 factor preparation, consisting of both supercoil and relaxed circular forms (Fig. 2a) was quantitatively converted by either Eco RI or Sma R into the open linear form (mass, 4.2 Md) (Fig. 2b, c); the sites of cleavage by Eco RI and Sma R were distinct and different from each other because digestion of the colicin E1 factor preparation sequentially by Eco RI and Sma R produced two distinct fragments (Fig. 2d). The approximate positions of the two cleavage sites relative to each other (Fig. 1) will be discussed below.

The undigested RSF 1010 preparation, consisting of both supercoil and relaxed circular forms (Fig. 2e), was quantitatively converted to the linear form (mass, 5.5 Md) by Eco RI (Fig.

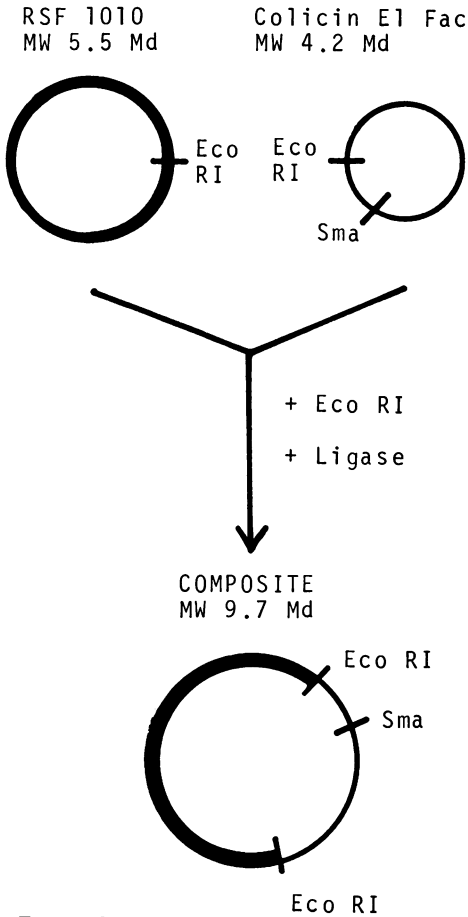

FIG. 1. *Schematic outline of assembly of the composite plasmid, including relevant sites of cleavage with restriction enzymes.*

2f), whereas only a trace amount of linear form was seen in the Sma R-treated sample (Fig. 2g). We assume that the small amount of linear form seen arose from random cleavage by trace nuclease contamination. Sequential digestion by Eco RI and Sma R only produced a single fragment with mobility characteristic of the linear form (mass, 5.5 Md) (Fig. 2h).

The preparations of RSF 1010 and colicin E1 factor including their respective linear forms prepared with Eco RI as well as a set of standard fragments obtained from phage λ (cb2) DNA with Eco RI are shown in Fig. 3a through e. A mixture of linear RSF 1010 and colicin E1 factor prepared from their respective circular forms and co-electrophoresed is shown in Fig. 3f. The composite plasmid preparation shown in Fig. 3g consisted primarily of supercoils with a trace of the relaxed circular form and a smaller trace of the linear form. Treatment of the composite factor with Eco RI yielded two fragments indistinguishable from the fragments shown in Fig. 3f; these fragments (Fig. 3h) are therefore presumed to be linear RSF 1010 and linear colicin E1 factor, respectively. Digestion of the composite with Sma R produced a single linear molecule (Fig. 3i) with the mobility expected of linear DNA (mass, 9.7 Md). From the data presented in Fig. 2, we would expect a single cleavage site for Sma R that would occur in the colicin E1 factor portion of the composite plasmid. This is confirmed by sequential digestion of the composite plasmid with Sma R and Eco RI (Fig. 3j), in which the mobility of the presumptive RSF 1010 fragment was unchanged whereas the presumptive colicin E1 factor was absent, and in its place appeared two fragments comparable to those obtained by digestion of colicin E1 factor with Sma R (Fig. 2d).

Eco RI and Sma R introduced single cuts in the colicin E1 factor. Digestion of the colicin E1 factor sequentially with Sma R and Eco RI

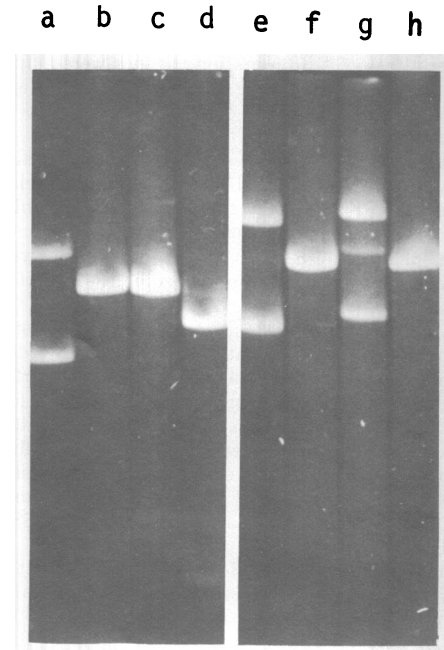

FIG. 2. *Cleavage of RSF 1010 and colicin E1 factor with Eco RI and Sma R endonucleases. Colicin E1 factor and RSF 1010 were digested with endonuclease (enzyme indicated in parentheses), and resultant digests were fractionated. Direction of migration is downward. (a) Colicin E1 factor, supercoils plus relaxed circles; (b) colicin E1 factor (Eco RI); (c) colicin E1 factor (Sma R); (d) colicin E1 factor (Sma R plus Eco RI); (e) RSF 1010; (f) RSF 1010 (Eco RI); (g) RSF 1010 (Sma R); (h) RSF 1010 (Sma R plus Eco RI).*

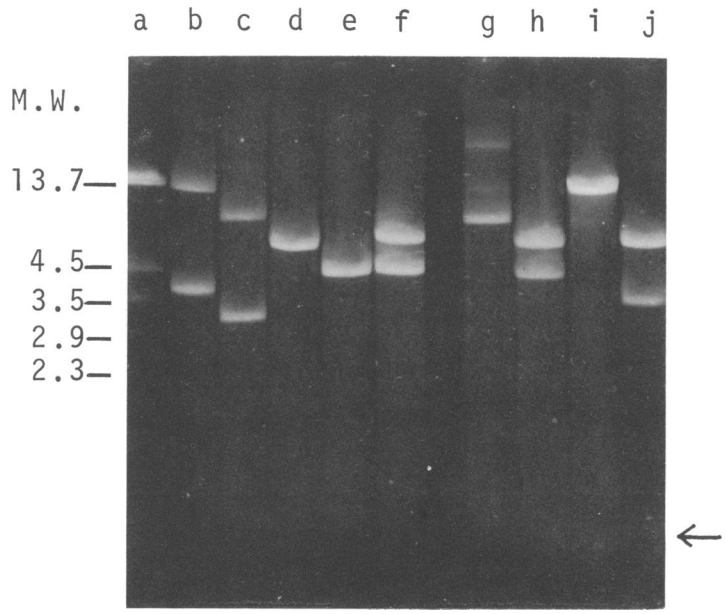

FIG. 3. *Cleavage of the composite plasmid and reference DNA samples with Eco RI and Sma endonucleases. The composite plasmid as well as reference DNA samples were digested with Eco RI and Sma R endonucleases as indicated. (a) λ (cb2) (Eco RI) standards; (b) RSF 1010, supercoils plus relaxed circles; (c) colicin E1 factor, supercoils plus relaxed circles; (d) RSF 1010 (Eco RI); (e) colicin E1 factor (Eco RI); (f) mixture of (d) plus (e); (g) composite plasmid, supercoil plus trace-relaxed circle and linear form; (h) composite plasmid (Eco RI); (i) composite plasmid (Sma R); (j) composite plasmid (Sma R plus Eco RI) (arrow indicates position of a faint faster-moving fragment derived from the colicin E1 factor as in Fig. 2d).*

resulted in two fragments (Fig. 2d). The mass of the major fragment was approximately 3.5 Md, and by subtraction the smaller fragment had an estimated mass of about 0.7 Md by comparison with the Eco RI fragments obtained from λ (cb2) DNA. This places the Sma R cut approximately 17% from the Eco RI cleavage site in colicin E1 factor or in the composite plasmid, 17% of the distance from one of the two Eco RI cleavage sites.

Biological properties of cells carrying the composite factor. The presence of RSF 1010 confers resistance to streptomycin and sulfonamide, whereas the presence of colicin E1 factor confers colicin E1 immunity as well as the synthetic capacity for colicin E1 synthesis. The composite factor confers streptomycin resistance, sulfonamide resistance, and colicin E1 immunity; however, colicin E1 production could not be detected (i.e., less 1% expected level) in mitomycin C-induced cells extracted with 10 mM sodium phosphate (pH 7.0)-buffered 1 M NaCl as described by Foulds and Barrett (4). Cells containing both colicin E1 factor and RSF 1010 separately had the same resistance properties but in addition synthesized colicin E1 (data not shown).

Replication of the composite plasmid in the presence of chloramphenicol. Colicin E1 factor continues to replicate for at least 6 h in the presence of chloramphenicol (2), whereas RSF 1010 does not (6). To establish the specificity of composite plasmid replication, 10-ml cultures were treated for varying lengths of time with chloramphenicol and controlled lysates prepared as described in stage 7. Equal portions of the lysate representing 2 ml of culture were precipitated with ethanol. The precipitates were collected and analyzed by agarose-ethidium bromide electrophoresis. The qualitative results (Fig. 4) show how the composite plasmid band can be visualized directly and that it appears to increase in the presence of chloramphenicol. When the plasmid band was labeled by addition of [^{3}H]thymidine to the medium, followed by addition of a [^{14}C]thymidine-labeled internal standard similar to the method described by Clewell (2) (Fig. 5), the specific activity of the plasmid band could be seen to increase for 6 h whereas the specific activities of RSF 1010 and chromosomal DNA did not.

From the similarity of these data to those of Clewell (2) and the report of Guerry et al. (6)

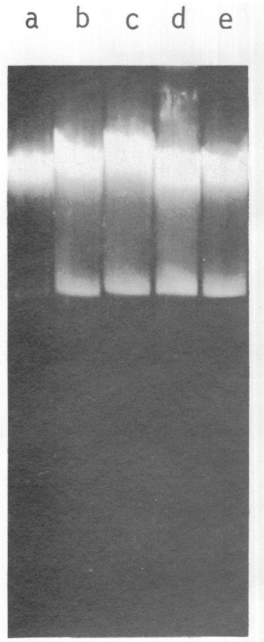

FIG. 4. *Replication of the composite plasmid in the presence of chloramphenicol; qualitative analysis by agarose-ethidium bromide electrophoresis. Controlled lysates were prepared from cultures grown in chloramphenicol for increasing lengths of time. Equal portions were fractionated by agarose-ethidium bromide electrophoresis. (a) through (e) show, respectively, 0, 2, 4, 6, and 9 h of growth in chloramphenicol.*

that RSF 1010 is not preferentially replicated in the presence of chloramphenicol, we assume that the colicin E1 factor controls the replication of the composite plasmid, although the application of addition criteria would be useful in testing this inference. The possible contribution of RSF 1010 to initiation of replication in the composite plasmid cannot be evaluated from these experiments. Regardless of the exact details of composite plasmid replication specificity, these data demonstrate the potential usefulness of the composite plasmid for amplification of DNA.

Partial digestion of the composite plasmid with Eco RI. From the previous experiments, use of the colicin E1 factor as replicator for heterologous DNA appears to confer certain advantages. Unfortunately, selection of transformants on the basis of their immunity to colicin E1 alone is difficult in our hands because of the apparent high rate of mutation to colicin E1 resistance. Replication of heterologous DNA by attachment to the composite plasmid could circumvent this difficulty if one could preferentially open only one of the two Eco RI sites. Such a form could be selected with a combination of streptomycin and colicin E1. The feasibility of preparing the 9.7-Md linear by partial digestion with Eco RI is shown in Fig. 6. After 20 min of digestion with Eco RI, further cleavage of the composite plasmid resulted in the appearance of the linear forms of colicin E1 factor and RSF 1010. The 9.7-Md linear can be purified by elution from the agarose gel and then utilized as replicator for the clonal selection and amplification of heterologous DNA.

DISCUSSION

This report describes the construction of a composite plasmid in which DNA from the R factor RSF 1010 has been covalently attached in vitro to the colicin E1 factor; the composite is replicated preferentially over chromosomal DNA in the presence of chloramphenicol. This mode of replication suggests a dependence on the replicator function of the colicin E1 factor constituent of the composite plasmid. These observations confirm the report of Hershfield et

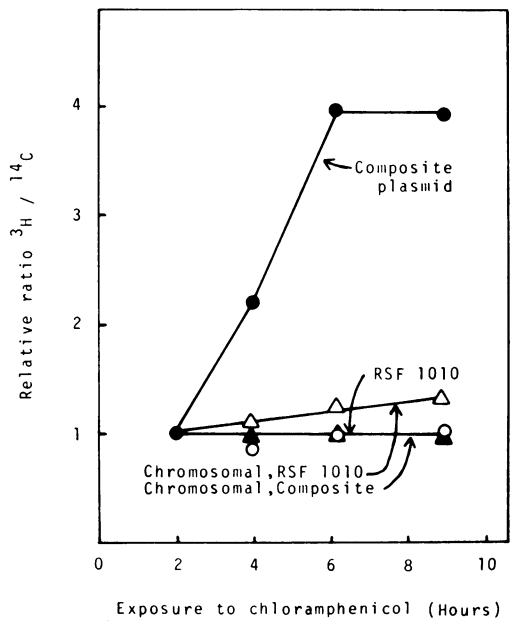

FIG. 5. *Replication of the composite plasmid in the presence of chloramphenicol; quantitative analysis of the [^3H]thymidine-labeled products. Controlled lysates containing composite plasmid and RSF 1010 were prepared from cultures grown in chloramphenicol as indicated and supplemented with [^3H]thymidine. Equal portions fractionated by agarose-ethidium bromide electrophoresis together with [^{14}C]thymidine-labeled plasmid DNA added as internal standard. The ratio $^3H/^{14}C$ was calculated and the data were normalized to a value of 1 for the 2-h point.*

al. (7) pertaining to construction of a composite plasmid in which the colicin E1 factor was used to replicate tryptophane synthetase genes of *E. coli*. In the case described above, a naturally occurring combination of nontransmissible antibiotic resistance genes has been attached to a new replicating unit. One advantage of the high levels of plasmid synthesis obtained from chloramphenicol-treated cultures is the relatively small physical scale (100 ml) required for preparation of useful quantities (25 to 100 μg) of plasmid. This type of methodological design is of importance in connection with containment and disposal of organisms that might be regarded as potential biohazards.

As a further consequence of plasmid replication in chloramphenicol-treated cultures, a sample of controlled lysate derived from as little as 1 ml of culture can be directly analyzed by gel electrophoretic techniques without using radioisotopes. This desirable property combined with the high sensitivity of analytic methods using fluorescence facilitates screening of large numbers of cultures in connection with studies on plasmids.

In these studies, the ligase reaction was also monitored by means of the agarose-ethidium bromide electrophoresis method. Results of this analysis clearly revealed the formation of a complex mixture of higher-molecular-weight products, some of which are presumed to be conformers of the composite plasmid, as well as polymeric forms of the constituent plasmid elements. A detailed analysis of the ligase reaction with respect to time and enzyme concentration dependence, as well as a demonstration of the array of ligase products formed from mixtures of differing degrees of complexity, has been prepared and will be published separately (T. Tanaka and B. Weisblum, unpublished data).

In the preparative procedure described above, composite plasmids formed in the ligase reaction were not purified before use in the transformation stage; rather, we used the unfractionated ligase reaction mixture and relied on double selection plus cloning of the selected transformants in order to effect the equivalent of a more laborious chemical fractionation step. Clearly, the cloning procedure is a very powerful purification step in which one of the two possible isomeric composite plasmids was selected; it is very unlikely that these two isomers could have been separated from each other by fractionation of the ligase reaction mixture by techniques based on the chemical or physical properties of these molecules.

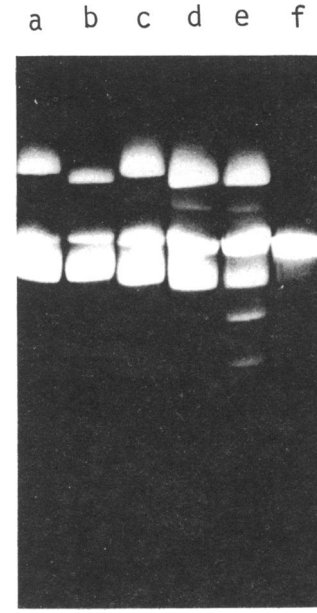

FIG. 6. *Conversion of the composite plasmid to the linear form by partial digestion with Eco RI. The composite plasmid was digested with threefold diluted Eco RI for varying lengths of time. A fraction with mobility corresponding to 9.2 Md appears first followed by appearance of the parental linears, colicin E1 factor and RSF 1010, after 20 min of incubation. (a) Undigested composite plasmid preparation; (b) Eco RI digestion for 2 min; (c) Eco RI digestion for 5 min; (d) Eco RI digestion for 10 min; (e) Eco RI digestion for 20 min; (f) Sma R linear of the composite plasmid.*

The precise physical conformation of the ligase product responsible for the transformation event remains unknown, since supercoil, relaxed circular, and linear conformers of the input plasmid can be presumed to be readily interchangeable. Regardless of the precise conformation of the ligase product that eventually manifested itself in the form of the composite plasmid, it was apparently formed in high enough yield by the procedures outlined above to effect the desired transformation. The action of DNA ligase is apparently required because two attempts at plasmid construction in which ligase was omitted from stage 2 failed to yield the desired composite factor. The level at which the reaction failed to yield desired product was not characterized further, and all subsequent constructions have been done with DNA ligase.

Conversion of the circular composite to its linear conformer can be effected by partial digestion with Eco RI such that only one of two susceptible sites is cleaved. By reiteration of the

preparative stages outlined above with the linear composite and an Eco RI digest of *Drosophila melanogaster* DNA as substrates for the ligase reaction, a chimeric plasmid capable of replication and chloramphenicol-induced amplification in *E. coli* has been constructed (T. Tanaka and B. Weisblum, unpublished data). Details of this construction including a map and proof of structure of the chimera will be presented separately.

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LITERATURE CITED

1. Allet, B., P. G. N. Jeppesen, K. J. Katagiri, and H. Delius. 1973. Mapping the DNA fragments produced by cleavage of λ DNA with endonuclease RI. Nature (London) **241**:120-123.
2. Clewell, D. B. 1972. Nature of col E1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667-676.
3. Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling. 1973. Construction of biologically functional plasmids *in vitro*. Proc. Nat. Acad. Sci. U.S.A. **70**:3240-3244.
4. Foulds, J., and C. Barrett. 1973. Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246: two new classes of tolerant mutants. J. Bacteriol. **116**:885-892.
5. Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064-1066.
6. Guerry, P., J. Van Embden, and S. Falkow. 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619-630.
7. Hershfield, V., H. W. Boyer, C. Yanofsky, M.A. Lovett, and D. Helinski. 1974. Plasmid col E1 as a molecular vehicle for cloning and amplification of DNA. Proc. Nat. Acad. Sci. U.S.A. **71**:3455-3459.
8. Mandel, M., and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159-162.
9. Mertz, J. E., and R. W. Davis. 1972. Cleavage of DNA by RI restriction endonuclease generates cohesive ends. Proc. Nat. Acad. Sci. U.S.A. **69**:3370-3374.
10. Sharp, P. A., B. Sugden, and J. Sambrook. 1973. Detection of two restriction endonucleases in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry **12**:3055-3063.
11. Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson. 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. J. Biol. Chem. **243**:4543-4555.