Replication of the Deoxyribonucleic Acid of Multiple-Drug-Resistance Factor in *Escherichia coli*

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1. It was shown that a system previously described for labelling R-factor DNA during transfer to an irradiated recipient strain of *Escherichia coli* did not allow high selectivity in the incorporation of thymine into R-factor DNA. 2. Lack of selectivity was shown to be due to cross-feeding from recipient to donor strain. 3. An improved system using a nalidixic acid-resistant recipient strain is described in which incorporation of thymine into the DNA of donor cells is minimized by addition of nalidixic acid after completion of transfer of the plasmid during conjugation.

Replication of plasmid DNA following transfer between bacterial cells has been studied in various ways that depend partly on the nature of the donor and recipient strains. In cases of transfer to a homologous recipient, it is difficult to distinguish between replication of the plasmid and the recipient chromosome, because of the similarity in base composition of their respective DNA. Freifelder & Freifelder (1968) devised a system for transfer of the plasmid F'-*lac* by using conditions that were believed to allow incorporation of isotopically labelled thymine only into the DNA of the plasmid after transfer to the recipient. This method has been recommended for the isolation of plasmid DNA (Freifelder, 1971) and has been adapted (Silver & Falkow, 1970) for the study of replication of R-factor after transfer to a recipient cell. Similar experiments on the replication of R-factor were carried out by the present authors (Barker *et al.*, 1971) with results that cast doubt on the interpretation of the earlier results of both Freifelder & Freifelder (1968) and of Silver & Falkow (1970) for the reasons outlined below.

The method of Freifelder & Freifelder (1968) involves transfer of the plasmid from a donor strain that is incapable of utilizing thymine by virtue of a genetic deficiency in thymidine kinase. The recipient is a thymine-requiring mutant, which is deficient in DNA repair processes. Before mating, the recipient is irradiated by u.v. light, which prevents replication of the chromosome. However, the irradiated cells are able to act as recipient, and incorporation of [³H]thymine into DNA during conjugation has been regarded as representing solely the replication of the newly transferred plasmid. In agreement with this, incorporation of [³H]thymine into DNA following mating is in excess of that observed in donor and irradiated recipient cultures incubated separately. In the present authors' experiments, controls were carried out by using mixed cultures of irradiated

recipient cells and cells of the donor strain lacking the R-factor. In such control experiments, incorporation of [³H]thymine into DNA was also in excess of that found in individual cultures incubated separately. This suggested that the excess incorporation observed by Freifelder & Freifelder (1968) and by Silver & Falkow (1970) was not due entirely to replication of a plasmid after transfer. The present paper describes experiments that show that the excess labelling is due partly to cross-feeding, which vitiates the system. A modified system is put forward in which the difficulty is minimized.

Materials and Methods

Reagents, antibiotics and radiochemicals

Reagents were AnalaR grade, either from BDH, Poole, Dorset, U.K., or from Hopkin and Williams, Chadwell Heath, Essex, U.K. Antibiotics and enzymes were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K.

Bacterial strains

All strains used were derivatives of *E. coli* K12. Strains DF85 (*pro*⁻, *met*⁻, BU^R, *lac*^{DEL}) and DF69 (*pro*⁻, *arg*⁻, *thy*⁻, *uvrA*⁻, *lac*⁻, *str*^R) were gifts from Dr. D. Freifelder (Brandeis University, Waltham, MA, U.S.A.). Strain DF85R1drd19 was obtained by conjugation of strain DF85 with strain J5-3 (*pro*⁻, *met*⁻, *lac*⁺) carrying the derepressed R-factor R1drd19, which was a gift from Dr. Naomi Datta (Royal Postgraduate Medical School, London W12 0HS, U.K.). Strain DF85R1drd19 was selected as *lac*⁻, chloramphenicol-resistant clones. Strain DF69nal^R was obtained by mutation of strain DF69 by using nitrosoguanidine. It was found that incorporation of [³H] thymine into DNA of this strain is

inhibited by only 4–5% by 50 µg of nalidixic acid (1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid)/ml, whereas similar incorporation by *E. coli* DF85 is inhibited to the extent of 95% by the same concentration of the drug.

Growth of organisms and conjugation

Incubations were at 37°C using the minimal salts medium of Freifelder & Freifelder (1968) supplemented with 5 mg of casein hydrolysate (BDH), 2 mg of glucose, 40 µg of each of L-proline, L-arginine and L-methionine and 1 µg of thymine/ml. This is referred to as supplemented FMS medium. Conjugal transfer of plasmid was carried out by incubation with gentle shaking of mixtures of equal volumes of exponential cultures (E_{650} approx. 0.3) of donor and recipient strains. For experiments using irradiated recipient cells, cells were grown as described above, harvested by filtration (Oxoid membrane filter, pore size 0.4 µm), washed with FMS medium at 0°C and resuspended in the same medium at 4°C. Samples (20 ml) of the cell suspension were irradiated in a sterile Petri dish by using an unmasked Chromatolite u.v. lamp (Heracus Quartz Fused Products, Weybridge, Surrey, U.K.) set at 15 cm (u.v. dose = $6.1 \text{ J} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ according to the manufacturer's figure). After irradiation, the cell suspensions were warmed to 37°C and growth requirements were added to give concentrations corresponding to supplemented FMS medium.

Selective media were prepared by addition of 2% (w/v) agar and 2% (w/v) glucose to minimal salts medium (67 mM- K_2HPO_4 /30 mM- KH_2PO_4 /56 mM- Na_2SO_4 / 50 mM- NH_4NO_3 / 37 mM- NH_4Cl / 16 mM- MgSO_4 , pH 7.2). For selection of strain DF85, 20 µg each of L-proline and L-methionine/ml were added. For selection of strain DF69 and derivatives, 250 µg of streptomycin and 20 µg each of thymine, L-proline and L-arginine/ml were added. For selection of strains carrying the R-factor R1drd19, 20 µg of chloramphenicol/ml were added to the appropriate medium.

Measurement of acid-insoluble radioactivity

Samples of cultures (0.5 or 1.0 ml) were added to 5 ml of 10% (w/v) trichloroacetic acid and 0.1 ml of bovine serum albumin (2 mg/ml) was added. After being allowed to stand for 1 h at 4°C, precipitates were collected on glass-fibre discs (Whatman GF/C, 2.1 cm diam.), washed twice with 10% trichloroacetic acid followed by ethanol and diethyl ether, and dried. The discs were immersed in 5 ml of scintillation fluid, consisting of 0.4% 2,5-diphenyloxazole and 0.04% 1,4-bis-(4-methyl-5-phenoxazol-2-yl)benzene in xylene, and counted for radioactivity in the Packard Tri-Carb series 3001 liquid-scintillation spectrometer.

Characterization of DNA

Cells were grown in presence of the appropriate labelled precursor, harvested by filtration by using an Oxoid membrane filter (pore size 0.4 µm), washed and resuspended in 1 ml of buffer containing 0.5 mM-EDTA, 0.5 M-Tris/HCl, pH 8.0, to which was added 0.1 ml of lysozyme (2 mg/ml). After incubation at 45°C for 10 min, 0.1 ml of sodium dodecyl sulphate (100 mg/ml) was added and incubation was continued for a further 10 min. DNA was sheared by pipetting 20 times in and out of a 1 ml pipette. (0.5 mm internal diam.). Lysates were cooled to 0°C and examined immediately either by equilibrium centrifugation in the presence of CsCl dissolved in buffer containing 50 mM-Tris/HCl, 50 mM-EDTA and 50 mM-NaCl, pH 8.0 ($n = 1.401$), and ethidium bromide (300 µg/ml), or by sedimentation in a 5–20% (w/v) sucrose gradient containing 10 mM-EDTA and 0.3 M-NaOH. Samples were withdrawn from the bottom of the tubes.

Results

Transfer of R-factor from E. coli DF85R1drd19 to E. coli DF69 and subsequent radioactive labelling

Initial experiments were carried out essentially as described by Silver & Falkow (1970). A suspension of *E. coli* DF69 (5 ml; approx. 2×10^8 cells/ml), irradiated with u.v. light as described above, was incubated with 5 ml (approx. 1×10^8 cells/ml) of a culture of *E. coli* DF85R1drd19, together with [methyl- ^3H]thymine (1 µg/ml; 7.9 Ci/g). Samples were removed at intervals and acid-insoluble radioactivity was measured. Similar measurements were made with *E. coli* DF85R1drd19 and irradiated *E. coli* DF69 incubated separately. The results are shown in Fig. 1(a). The results of a further series of experiments in which cultures of *E. coli* DF85R1drd19 were replaced by strain DF85 lacking the R-factor are shown in Fig. 1(b).

In order to characterize the material labelled with ^3H , *E. coli* DF85R1drd19 and irradiated cells of *E. coli* DF69 were incubated together with [methyl- ^3H]thymine as described above. A sample was removed after 30 min, the cells were harvested and lysed as described above, mixed with a similar lysate prepared from *E. coli* DF69R[−] grown in the presence of [^{14}C]thymine and centrifuged to equilibrium in CsCl in the presence of ethidium bromide. Fractions from the gradient were analysed for radioactivity and the results are shown in Fig. 2. In addition to a major ^3H -labelled component having the same buoyant density as DNA prepared from *E. coli* DF69R[−] grown in the presence of [^{14}C]thymine, there is a component having a higher buoyant density and comprising approx. 3.4% of the total ^3H -labelled material. This material, indicated by the arrow, is characteristic of covalently closed circular DNA (Radloff *et al.*, 1967).

Fig. 1. Incorporation of radioactivity into acid-insoluble material during incubation of mixed cultures of (a) *E. coli* DF85R1drd19 and DF69, and (b) *E. coli* DF85R⁻ and DF69

(a) Equal volumes of an exponentially growing culture of *E. coli* DF85R1drd19 and a culture of *E. coli* DF69 irradiated with u.v. light were mixed and incubated in the presence of [methyl-³H]thymine. Similar incubations were also carried out with the two cultures separately. Samples (1 ml) were withdrawn at intervals, precipitated with trichloroacetic acid and the precipitates analysed for radioactivity as described in the text. (b) The experiment was carried out as described for (a) except that *E. coli* DF85R⁻ was used instead of strain DF85R1drd19. ●, Acid-insoluble radioactivity from mixed cultures; ▲, incorporation in strain DF85R1drd19 or DF85R⁻ incubated alone; ■, incorporation in irradiated strain DF69 incubated alone.

The above results indicate that some labelling of R-factor DNA occurs during incubation of donor cells with irradiated recipient cells. However, since excess labelling in mixed cultures occurs when no R-factor-containing cells are present, it follows that incorporation of radioactivity is not confined to plasmid DNA in the recipient as assumed by Silver & Falkow (1970).

Origin of [methyl-³H]thymine incorporation during incubation of *E. coli* strain DF85R⁻ with u.v.-irradiated strain DF69

To investigate the origin of the ³H incorporation into acid-insoluble material observed during incubation of *E. coli* DF85R⁻ with u.v.-irradiated strain DF69 (Fig. 1b), cells of strain DF69 were irradiated with 850 J/m² of u.v. radiation and were resuspended in supplemented FMS medium containing 80 μCi of [methyl-³H]thymine (specific radioactivity 10 Ci/g).

Half of the culture (10 ml) was centrifuged immediately (10000g for 10 min) to remove the cells (supernatant A). The remaining portion was incubated at 37°C for 120 min and the cells removed by centrifugation as above (supernatant B). Samples of each supernatant (1 ml) were separately inoculated with 1 ml of an exponential culture of *E. coli* DF85R⁻ and incubated for 60 min. After 120 min, samples (0.2 ml) were withdrawn from each and incorporation of radioactivity into acid-insoluble material measured as described above. Corresponding samples from supernatant A and supernatant B gave 300 and 1297 c.p.m. of ³H respectively, indicating that incubation with the u.v.-irradiated cells had modified the radioactively labelled thymine to make it more readily incorporated into acid-insoluble material by the *E. coli* DF85R⁻ cells.

Chromatography of samples of supernatant B with aq. butan-1-ol as solvent and with aq. NH₃ in the

Fig. 2. Identification of covalently closed circular DNA by dye-buoyant-density equilibrium centrifugation

Conjugation was effected between *E. coli* DF85R1drd19 and irradiated strain DF69 in the presence of [methyl- ^3H]-thymine. A sample of the culture was taken after incubation for 30 min, lysed, and the DNA centrifuged to equilibrium in the presence of CsCl and ethidium bromide together with a sample of DNA prepared from *E. coli* DF69R⁻ grown in the presence of [^{14}C]-thymine. Fractions from the gradient were analysed for ^3H (●) and ^{14}C (▲). (Note change of scale indicated by broken line.) The component containing covalently closed circular DNA is indicated by the arrow.

bottom of the tank, following the method of Wyatt (1955), revealed two radioactive components, one of which remained at the origin, the other moving at the same rate as the standard sample of thymine ($R_f = 0.47$). Supernatant A was found to contain only the [^3H]-thymine component when chromatographed similarly. The material from supernatant B, which remained at the origin, was shown to be [^3H]-dTMP in the following way. The material was eluted with ice-cold 95% (w/v) formic acid, evaporated to dryness *in vacuo*, and taken up in 1 ml of glycine/ Mg^{2+} buffer (Heppel & Hilmoe, 1955). A sample of the material had the same R_f value as an authentic sample of dTMP when chromatographed on paper using propan-2-ol/ HCl /water (170:41:39, by vol.) as solvent system. After incubation at 37°C for 60 min with snake venom, following the method of Heppel & Hilmoe (1955), the material chromatographed faster than thymine and at the same rate as thymidine, using the butan-1-ol/aq. NH_3 system. After hydrolysis with 95% (v/v) formic acid at 175°C for 30 min the material had the same R_f as standard thymine.

In order to demonstrate the utilization of dTMP by cells of *E. coli* strain DF85R⁻, the [^3H]-dTMP identified in supernatant B was separated from [^3H]-thymine by adsorption on to activated charcoal and was eluted with ethanol/aq. NH_3 (sp.gr. 0.88)/water (25:1:24, by vol.). The material was evaporated to dryness under reduced pressure at 50°C and taken up in 2 ml of water. Duplicate samples (0.5 ml) of an exponentially growing culture of *E. coli* DF85R⁻ were prepared. To one culture was added 1.45 ml of supplemented FMS medium containing $3.9\mu\text{Ci}$ of [^3H]-thymine, and to the other culture was added the same volume of supplemented FMS medium, but containing $3.9\mu\text{Ci}$ of the purified [^3H]-dTMP isolated from supernatant B. The cultures were incubated for 120 min at 37°C , and samples (0.25 ml) were assayed for acid-insoluble radioactivity as described above. The cells were found to incorporate 1×10^3 c.p.m. of ^3H from [^3H]-thymine and 4.9×10^4 c.p.m. from [^3H]-dTMP.

These results indicate that labelling of DNA in donor cells by thymine can take place in mixed cultures of *E. coli* DF85 and *E. coli* DF69 by a process of cross-feeding which by-passes the genetic block in thymine utilization in strain DF85. In order to avoid the effects of cross-feeding and in an attempt to improve the selective labelling of R-factor after transfer to irradiated cells, a system was devised using nalidixic acid to prevent replication of DNA in strain DF85 cells after completion of the transfer of R-factor to the recipient strain DF69.

Nalidixic acid specifically inhibits synthesis of DNA by a mechanism that is not understood. By using a nalidixic acid-resistant recipient (*E. coli* DF69^{nal^R}), it was anticipated that replication of the R-factor after transfer to this strain would not be inhibited by the drug. This expectation was made more likely by experiments which showed not only that, in the absence of the drug, *E. coli* DF69^{nal^R} is an efficient recipient for the plasmid R1drd19, but also that, in the presence of the drug, *E. coli* DF69^{nal^R} carrying plasmid R1drd19 is able to donate it to a second nalidixic acid-resistant recipient. The latter observation indicated that DNA synthesis is operative in *E. coli* DF69^{nal^R} in presence of the drug. Since it is known that nalidixic acid prevents conjugal transfer of DNA from sensitive strains by virtue of its inhibitory action on DNA replication (Barbour, 1967; Fisher & Fisher, 1968; Hane, 1971), in the experiments described below, nalidixic acid was added after completion of the transfer of the R-factor from the sensitive strain DF85R1drd19 to the resistant recipient strain DF69^{nal^R}.

Transfer of R-factor from E. coli R85R1drd19 to E. coli DF69^{nal^R} and subsequent radioactive labelling

Preliminary experiments showed that, in mixed cultures of *E. coli* DF85R1drd19 and *E. coli* DF69^{nal^R},

Fig. 3. Incorporation of radioactivity into acid-insoluble material during incubation of mixed cultures of *E. coli* DF85R1drd19 and DF69nal^R, and *E. coli* DF85R⁻ and DF69nal^R, in the presence of nalidixic acid

(a) Experiments were carried out as described for Fig. 1 except that strain DF69nal^R was used in place of DF69 and that nalidixic acid (final concentration 50 $\mu\text{g/ml}$) was added after 20 min incubation. Acid-insoluble radioactivity was measured in mixed cultures containing strain DF85R1drd19 (●) and mixed cultures containing strain DF85R⁻ (■). Experiments were also carried out with the same mixed cultures that were rapidly stirred at intervals during incubation (○ and □). (b) Results obtained in three separate experiments as described in (a) are plotted to show, at various times of incubation, the difference between incorporation in mixed cultures with and without plasmid transfer expressed as a percentage of that difference at 30 min incubation. ▲, Mean of values obtained in the three experiments. The vertical lines indicate the maximum variation between results.

transfer of the plasmid is complete in approx. 20 min.

An exponentially growing culture of *E. coli* DF85R1drd19 was added to an equal volume of an irradiated culture of *E. coli* DF69nal^R and incubated with [*methyl*-³H]thymine as described above and samples were withdrawn at intervals for measurement of acid-insoluble radioactivity. After 20 min, 50 μg of nalidixic acid/ml were added and incubation and removal of samples were continued. Similar experiments were carried out simultaneously in which *E. coli* DF85R1drd19 was replaced by *E. coli* DF85. Parallel experiments were also carried out using conditions identical with those described above except that the cultures were subjected to brief rapid stirring every 5 min to reduce conjugation. The results in Fig. 3(a) are typical of three series of experiments and show an excess labelling of DNA in mixed cultures containing R-factor compared with similar

cultures lacking R-factor. Moreover, the excess labelling is abolished by vigorous stirring of the mixed cultures. The excess labelling is thus associated with the transfer of R-factor and its progress with time is shown in three separate experiments in Fig. 3(b).

A mixed culture containing *E. coli* DF85R1drd19 and irradiated *E. coli* DF69nal^R was grown in the presence of [*methyl*-³H]thymine and nalidixic acid as described above. A sample was taken after 40 min incubation (20 min after addition of nalidixic acid) and, after addition of a solution of DNA prepared from *E. coli* DF69 grown in the presence of [¹⁴C]-thymine, was examined for covalently closed circular DNA by centrifugation in the presence of CsCl and ethidium bromide. The results shown in Fig. 4 show the presence of a fraction, indicated by the arrow, amounting to approx. 1.6% of the total ³H-labelled

Fig. 4. Identification of covalently closed circular DNA by dye-buoyant-density equilibrium centrifugation in the presence of nalidixic acid

Experiments were carried out as described for Fig. 2 except that *E. coli* DF69^{nal} was used as recipient and 50 µg of nalidixic acid/ml were added after 20 min incubation. ¹⁴C-labelled DNA from *E. coli* DF69 was used as a density marker and fractions were analysed for ³H (●) and ¹⁴C (▲) as described previously. (Note change of scale indicated by broken line.) The component containing covalently closed circular DNA is indicated by the arrow.

DNA, banding at the higher density expected of covalently closed circular DNA. This component is absent from the ¹⁴C-labelled DNA.

In order to examine in more detail the formation of covalently closed circular DNA after completion of the incorporation of thymine into plasmid DNA, mixed cultures were incubated with [*methyl-³H]-thymine as described above and samples were taken after 20, 30 and 60 min incubation (that is, 0, 10 and 40 min after addition of nalidixic acid) and centrifuged in alkaline sucrose gradients. No fast-sedimenting component corresponding to covalently closed circular DNA was observed in the sample taken after 20 min incubation (that is, the same time*

Fig. 5. Sedimentation of DNA in an alkaline sucrose gradient

Conjugation was effected between *E. coli* DF85R1drd19 and strain DF69^{nal} in presence of [*methyl-³H]-thymine as described for Fig. 4. Incubation was continued for 60 min and a sample of the culture, after lysis as described in the text, was centrifuged in an alkaline sucrose gradient and fractions were analysed for ³H. (Note change of scale indicated by broken line.) The component containing covalently-closed circular DNA is indicated by the arrow.*

as the addition of nalidixic acid). A very small fast-sedimenting fraction was observable after 30 min. Fig. 5 shows the distribution of radioactivity in the gradient after 60 min incubation and the presence of a fast-sedimenting fraction indicated by the arrow.

Discussion

The results of experiments carried out essentially as described by Silver & Falkow (1970) indicate that, under their conditions, some covalently closed circular DNA is labelled by [³H]-thymine. However, in our experiments, since labelling of DNA is greater in mixed cultures than that in separate cultures whether or not transfer of R-factor occurs, neither the excess labelling nor the labelled covalently closed circular DNA can be ascribed wholly to plasmid DNA replication in the recipient. The demonstration that labelled thymine is converted in the mixed cultures into dTMP and that this is capable of being used by *E. coli* DF85 for synthesis of DNA indicates that, under the conditions used by Silver & Falkow (1970), labelling of both plasmid and chromosomal DNA may occur in the donor cells. The cross-feeding is presumed to arise, because of the inability of the irradiated cells to utilize dTMP formed from the exogenously supplied thymine for synthesis of chromosomal DNA and is therefore to be expected also in the experiments of Freifelder & Freifelder (1968).

Consequently, in neither case is labelling to be regarded as confined to plasmid DNA.

In the modified mating system described in the present paper, it is possible selectively to inhibit semiconservative replication of DNA in the donor cells with nalidixic acid. However, it is necessary to delay addition of the drug until after completion of the transfer of R-factor, since conjugal transfer of plasmid is dependent on DNA replication in the recipient (see above). Consequently, in the early stage of the incubation, before addition of nalidixic acid, some incorporation of radioactivity into DNA in the donor cells involving cross-feeding of dTMP is possible. After addition of nalidixic acid, it is to be expected that semiconservative synthesis of DNA in the donor will virtually stop. However, nalidixic acid is without effect on repair synthesis of DNA (Simon *et al.*, 1974) and hence incorporation of radioactivity into plasmid DNA in the donor may possibly take place to a small extent. Thus the expected effect of adding nalidixic acid to the mixed cultures of donor and irradiated, nalidixic acid-resistant recipient is largely to limit incorporation of radioactivity to plasmid DNA in the recipient and to prevent further transfer of R-factor.

In agreement with the above predictions, it is seen from Fig. 3(a) that in the mixed culture containing no R-factor there is some incorporation of thymine into DNA during the first 20 min that is attributable to DNA replication in the donor. However, addition of nalidixic acid decreases this incorporation to a low background level. This is in contrast with the continued incorporation in the analogous experiments in which nalidixic acid was not added (Fig. 1b). Fig. 3(a) shows that incorporation of thymine into DNA in mixed cultures in which R-factor transfer is operative is greatly in excess of that unassociated with plasmid transfer and that this difference is abolished by prevention of R-factor transfer by rapid stirring. It must be assumed that initial rapid incorporation represents largely R-factor DNA synthesis together with a small amount of labelling of chromosomal DNA in the donor. It is significant that in the mixed cultures

in which transfer of plasmid takes place, incorporation of thymine into DNA continues at a high rate for a short time after addition of nalidixic acid before being reduced to the background rate. It is concluded that the conditions described allow the formation of labelled plasmid DNA with minimal contamination by chromosomal DNA. It appears that, in the irradiated recipient cells, synthesis of DNA is complete within approx. 25 min of the start of conjugation (see Fig. 3). Formation of covalently closed circular DNA is demonstrable within 60 min of the start of conjugation (see Fig. 5) and the modified system described is suggested as suitable for the isolation of plasmid DNA and related purposes and is considered to overcome the objections discussed above in the method described by Freifelder (1971).

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