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Segregation of Genes for Nitrogen Fixation into Minicells of *Escherichia coli* (39806)

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Introduction. The nitrogen fixation (nif) genes of *Klebsiella pneumoniae* have recently been inserted into *Escherichia coli* F'- and R-factor plasmids (1-3). Because expression of the *nif* genes and the identity of at least one of the proteins involved are still incompletely understood, the following study was undertaken to incorporate a *nif*-containing plasmid into minicells of *E. coli* and to demonstrate expression of the hybrid plasmid in the minicells.

Minicells have been employed by several investigators to study replication, transcription, and translation of plasmid DNA (4). Since minicells do not contain chromosomal DNA, they will be a valuable aid for identifying and isolating plasmid-specific proteins which in normal cells would be obscured by the large and diverse amount of chromosomally directed protein synthesis.

Materials and methods. The bacterial strains used are shown in Table I. Rifampicin resistance was obtained by plating a suspension of χ984 onto agar medium containing 25 μg/ml of rifampicin. The diaminopimelic acid (DAP) auxotroph was constructed according to the rationale of Bukhari and Taylor (5).

Media used were L broth (6), Difco nutrient broth and nutrient agar, and minimal medium according to the formula of Phares (7). Nitrogen-free minimal medium (NFM) consisted of Phares' formula with KCl substituted mole for mole for NH_4Cl. All amino acids, vitamins, nucleic acid bases, drugs, and DAP were obtained from Sigma Chemical Co. Deoxyribonuclease I (DNase), B grade, and Pronase, B grade, were obtained from Calbiochem. Ribonuclease A (RNase), Type 1A, was from Sigma. Radioactive chemicals were obtained from New England Nuclear and PCS liquid scintillation cocktail from Amersham/Searle.

Bacterial conjugations were conducted under conditions described by Curtiss *et al.* (8). Donor recipient ratios were approximately 1:100. For experiments with minicell donors, purified AL6 donor cells and minicells were incubated in L broth for 45 min at 37° before addition of recipient (AL4) cells. This preincubation without DAP served to lyse about 90% of contaminating cells in the minicell suspension.

Nitrogen fixation ability was assayed by the acetylene reduction method described by Postgate (9). The assay medium consisted of NFM medium with 0.5% glucose, 10^{-4} *M* serine, and necessary vitamin and amino acid supplements in appropriate concentrations (8) depending on nutritive requirements of the strains used (Table I). Each assay tube (30-ml total volume) contained 6 ml of medium and 0.6 ml of bacterial inoculum. Acetylene (0.5 ml) was generated from calcium carbide and water and was injected after anaerobiosis was established. Nitrogen gas (10 ml) was injected to assure positive pressure. The tubes were then incubated for 12 hr or longer at 30°. Gas aliquots of 1 ml were removed and analyzed by gas chromatography using Porapak R (Supelco) molecular sieve packing. Protein was measured by the method of Lowry *et al.* (10).

Minicells were purified according to Roozen *et al.* (11) with a modification involving the use of "freeze-thaw" sucrose gradients (12). These gradients were constructed by freezing 35-ml aliquots of a sterile solution of 15% sucrose in NFM medium upright in sterile centrifuge tubes. The solutions were then thawed upright at room temperature. The gradients formed were almost linear (Glen Germain, personal communication) and allowed good separation of minicells from parent cells. Purity of 10^5 minicells per contaminating parent cell was obtained.

DNA labeling was accomplished by overnight incubation of bacterial cultures in nu-

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TABLE I. BACTERIAL STRAINS.

Designation	Genotypic and phenotypic characteristics	Plasmid	Origin
JC5466/RP41	*trp his rec*A46 Spc^R	RP41 (Km^R Tc^R $Carb^R$ *gnd*$^+$ *his*$^+$ *nif*$^+$ *shi*A$^+$)	R. A. Dixon, ARC Unit of Nitrogen Fixation, University of Sussex (3)
χ984	*minA minB purE pdxC his xyl ilv cyc*A *cyc*B *met* Str^R T_6^S T_3^R λ^-	–	R. Curtiss, III, University of Alabama (4)
AL4	χ984 Rif^R	–	Spontaneous mutant of χ984 selected on rifampicin
AL5	AL4 *dap*	–	NTG mutagenesis of AL4
AL6	AL4 *dap*	RP41	JC5466/RP41 × AL5

trient broth containing 5 μCi/ml of [*methyl*-^{14}C]thymidine (55 Ci/mmole). Minicells were isolated from late exponential or early stationary phase cultures. The final minicell pellet was resuspended in standard saline citrate buffer with 27% sucrose (13). Aliquots of 100 μl were precipitated with cold trichloroacetic acid (TCA) and counted according to Roozen *et al.* (11). In order to confirm that the [^{3}H]thymidine label was present in DNA, the minicells were lysed with detergent and pronase according to the method of Levy (13). An equal volume of 0.1 *M* $MgCl_2$ in 0.1 *M* phosphate buffer, pH 7.0, was added. Minicell lysates were incubated overnight at 37° with no further additions or with the addition of a few grains of RNase or DNase. Aliquots of 100 μl were precipitated with TCA and counted.

Protein synthesis assays were carried out as follows. Culture manipulations described by Levy (14) were used to obtain cultures with a maximum proportion of freshly formed minicells. After purification, the minicell pellet was suspended in NFM medium containing 0.5% glucose, adenine, pyridoxine, and all amino acids except leucine, glutamine, and asparagine. The absorbance of minicell suspensions at 530 nm was 0.72. Assay suspensions contained 0.5 ml of minicell suspension and 50 μCi of [^{3}H]leucine (79.8 Ci/mmole, 1 μCi/μl), and were incubated at 37°. Aliquots of 50 μl were removed from the tubes at various time intervals, precipitated with hot TCA, and counted according to Roozen *et al.* (11).

Results. Bacterial conjugation between JC5466 (RP41) and AL5 gave rise to His^+Rif^R colonies. One of these (AL6) was purified and assayed for minicell production and acetylene reduction. Both of these tests were positive and all phenotypic markers of the χ984 strain were present.

Two methods were used to demonstrate presence of the plasmid in minicells. Table II shows results of assays of acid-precipitable [^{3}H]thymidine in minicells at AL6 and the plasmid-free strain AL4. The RP41 plasmid is estimated to be 60×10^6 daltons (R. A. Dixon, personal communication). Since the molecular weight of the *E. coli* chromosome is 2.8×10^9 daltons (15), the plasmid is approximately 2.1% of its size. Table II shows that, assuming similar thymidine contents and labeling processes in the two kinds of DNA, each minicell contained approximately 4% of the chromosomal amount of DNA. Thus each minicell contained at least one and possibly two copies of the RP41 plasmid. As a further test of the presence of DNA, minicells were lysed and treated with DNase. TCA-precipitable counts were 80% decreased by DNase but not at all by RNase or pronase.

Conjugation experiments using minicell donors afforded proof that the minicells contained the specific *nif* genes. Cells from various stages in the minicell isolation procedure were also used as donors. Table III shows the results of these experiments. The efficiency of plasmid transfer by minicells (shown as recombinants/minicell), while lower by a factor of 60 than the efficiency of the whole culture, remained constant when the minicells were diluted and when viable cells were added to a concentration of 4 or 40 times that of the contaminating donor cells present in the minicell suspension. These results compare favorably with those of Levy and Norman (16), who studied transfer of the drug resistance plasmid, R

factor 222, by minicell donors. On the other hand, the apparent efficiency when expressed in terms of viable cells (numbers in parentheses) increased significantly as the cell-to-minicell ratio was decreased. This proved that the cells were not responsible for many of the recombinants.

The histidine operon genes carried on the plasmid were used for selection in the conjugation experiments described above. In order to prove that *nif* genes were also present, 11 recombinant colonies were picked at random from the selective agar plates and assayed for acetylene reduction. After 24 hr of incubation at 37°, all 11 cultures produced ethylene (500 ± 120 nmole/mg of protein, mean ± SE). Controls with plasmid-free strains exhibited negligible amounts of ethylene production (1 nmole/mg of protein).

TABLE II. INCORPORATION OF TCA-PRECIPITABLE [^{3}H]THYMIDINE INTO MINICELLS.

Cells	cpm/A_{530} unit	$\frac{\text{cpm/minicell}^a}{\text{cpm/cell}} \times 100$
AL6 cells (from low-speed pellet)	44,740	–
AL6 minicells (first determination)	14,170[b]	4.5
AL6 minicells (second determination)	13,220[b]	4.2
AL4 minicells	576[b]	0.2

[a] Assuming number of minicells/A_{530} unit = 7 × number of cells/A_{530} unit (15).

[b] Background and counts due to contaminating cells were subtracted.

Protein synthesis by minicells containing the RP41 plasmid was assayed in order to determine whether minicells could express genes present on the plasmid. Table IV shows the incorporation of [^{3}H]leucine into acid-precipitable radioactivity as a function of time after the addition of the labeled amino acid to suspensions of AL6 and AL5 minicells. The plasmid-containing minicells showed greater protein synthesis than did the AL5 minicells. The synthesis in plasmid-free minicells has been shown by Levy (17) to be due to long-lived messenger RNA present in the minicells at the time they are formed.

Protein synthesis by AL6 minicells incubated anaerobically was also observed, although at a much reduced rate.

Discussion. The experiments reported here have demonstrated that *E. coli* minicells do incorporate plasmids containing the

TABLE IV. INCORPORATION OF TCA-PRECIPITABLE [^{3}H]LEUCINE INTO MINICELLS.

Minicells	cpm × 10^{-3} after 60 min[a]	cpm × 10^{-3} after 180 min[a]
AL6 minicells	87.8	255.0
AL5 minicells	35.4	73.0
AL6 minicells incubated anaerobically[b]	6.28	n.d.
AL6 minicells + 10^{-3} *M* puromycin	1.88	3.30

[a] Counts due to contaminating cells were less than 5%.

[b] Anaerobiosis was achieved by bubbling with argon for 5 min before and during incubation with the label.

TABLE III. TRANSFER OF RP41 PLASMID FROM AL6 MINICELL CONJUGAL DONORS TO AL4 CELL RECIPIENTS.

Donor	Minicells/ml[a]	Viable cells/ml[b]	Recombinants/ml[c]	Recombinants/cell	Recombinants/minicell
Whole culture	–	1.3×10^6	2.4×10^4	0.018	–
Low-speed pellet (cells)	–	2.3×10^6	4.7×10^4	0.020	–
Low-speed supernatant	–	2.3×10^6	3.4×10^4	0.017	–
Sucrose-treated cells[d]	–	2.5×10^5	1.4×10^4	0.055	–
Sucrose-treated cells	–	2.5×10^4	0.1×10^4	0.040	–
Minicells	8.4×10^7	6.2×10^3	2.3×10^4	(3.7)	0.00027
Minicells	8.4×10^6	6.2×10^2	0.25×10^4	(4.0)	0.00030
Minicells + sucrose-treated cells	8.4×10^7	2.5×10^5	2.5×10^4	(0.100)	0.00030
Minicells + sucrose-treated cells	8.4×10^7	2.5×10^4	2.1×10^4	(0.840)	0.00025

[a] Assuming 7×10^9 minicells/A_{530} unit (15).

[b] Determined by plating suspensions containing no recipients on nutrient agar + DAP. This was carried out at the beginning of the 60-min mating incubation, so these are the highest possible numbers. (See Methods.)

[c] Selected on histidine-free, DAP-free agar plates. Neither donor nor recipient was able to grow on this medium.

[d] Pellet of the first sucrose gradient which was then centrifuged through a second gradient.

K. pneumoniae genes for nitrogen fixation. The presence of DNA in AL6 minicells was proved by the incorporation of acid-precipitable [^{3}H]thymidine in these minicells but not in minicells from an isogenic strain which did not contain plasmids. The function integrity of the RP41 plasmid was retained during passage through minicells and into new normal cells, as evidenced by the acetylene reduction ability of recombinants which received the plasmid from minicell donors.

The rate of protein synthesis in minicells which possessed the RP41 plasmid was much greater than that in minicells which did not contain plasmids. This indicated that plasmid-specific protein was synthesized, although no conclusions could be made as to the functional identity of this new protein.

Attempts to observe acetylene reduction by minicells have so far been unsuccessful due probably to the lag of 12 to 15 hr (18) before *nif* genes of *K. pneumoniae* are expressed. A method for shortening this lag time must be found which is compatible with minicell production. Experiments directed toward the achievement of this goal are presently underway

An additional note should be made concerning the technical utility of the Dap$^-$ mutant for experiments involving minicells. Incubation of minicell suspensions in medium containing lysine but not DAP results in lysis of contaminating parent cells (4). This is useful for controlling the multiplication of cells during conjugation experiments and during long incubation of minicell suspensions.

Summary. An *Escherichia coli* strain containing nitrogen fixation (*nif*) genes on a transferable plasmid (RP41) was conjugated with an *E. coli* minicell-producing strain. The *nif* genes were expressed in the new strain as measured by acetylene reduction assays. Segregation of the RP41 plasmid into minicells was proved by the incorporation of acid-precipitable [^{3}H]thymidine into minicells and also by the conjugal transfer of acetylene reduction ability during matings using minicell donors. Protein synthesis was measured by the time-dependent increase of acid-precipitable [^{3}H]leucine in minicell suspensions. Minicells which contained the plasmid had greater synthetic ability than those which did not.

Technical contributions to minicell purification were made. Sucrose gradients made by a new freeze–thaw procedure allowed easy preparations of sterile gradients which were used to obtain purity of 10^5 minicells per contaminating cell. A Dap$^-$ mutant was constructed in order to prevent outgrowth of contaminating cells during long incubations.

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