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Expression of drug resistance markers by spheroplasts of *Mycobacterium smegmatis* after fusion

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Abstract. Mycobacterial spheroplasts were prepared by treatment of the glycine-sensitized cells with a combination of lipase and lysozyme. They were stable for several hours at room temperature but were lysed on treatment with 0·1% sodium dodecyl sulfate. The spheroplasts could be regenerated on a suitable medium. Fusion and regeneration of the spheroplasts were attempted using drug resistant mutant strains of *M. smegmatis*. Recombinants were obtained from spheroplast fusion mediated by polyethylene glycol and dimethyl sulfoxide. Simultaneous expression of recombinant properties was observed only after an initial lag in the isolated clones. This has been explained as due to "chromosome inactivation" in the fused product.

Keywords. Spheroplast fusion; mycobacteria; prophage activation; recombination; chromosome inactivation.

Introduction

Generation of bacterial recombinants mediated by protoplast fusion has gained importance in recent years (Hopwood *et al* 1983). This method is particularly useful for those species of bacteria where other means of genetic transfer are not well-established. From the genetic studies on mycobacteria by several groups of workers it has generally emerged that even though the classical methods of gene transfer, viz. conjugation, transformation and transduction, operate in different species of mycobacteria they are often inefficient and irreproducible (Grange 1982). For this reason the protoplast fusion system may be of potential advantage in the study of mycobacterial genetics.

We had reported earlier a rapid and efficient procedure for the isolation of spheroplasts from *M. smegmatis*, using a combination of lipase and lysozyme (Sadhu and Gopinathan 1982). Rastogi *et al* (1983) using polyethylene glycol (PEG) have obtained spheroplast fusion in *M. aurum* yielding recombinants between strains differing in pigment production. In the present report we present observations on the expression of drug resistance markers by spheroplasts of *M. smegmatis* after fusion.

2. Materials and methods

Materials: Lysozyme, lipase, polyethylene glycol (PEG) and dimethylsulfoxide (DMSO) were from the Sigma Chemical Company, St. Louis, MO, USA. All other chemicals were of analytical grade.

Bacterial strains: *M. smegmatis* SN2 and the novobiocin- and kanamycin-resistant mutants of *M. smegmatis* SN2 were from our laboratory culture stocks.

Media (composition/litre): Minimal medium–(Karnik and Gopinathan 1980) Asparagine, 5·0 g; KH_2PO_4, 5·9 g; K_2SO_4, 0·5 g; $MgCO_3$, 0·5 g; citric acid, 1·5 g; glycerol, 20 ml; pH adjusted to 7·4. Tween-80 (0·3% v/v) was included to minimize clumping of mycobacteria. Regeneration medium–peptone, 10·0 g; beef extract, 3·0 g; NaCl, 5·0 g; casamino acids, 0·1 g; glucose, 10·0 g; K_2SO_4, 0·25 g; $CaCl_2$, $6H_2O$, 0·22 g; $MgCl_2$, $6H_2O$, 0·20 g. LB medium–Bacto tryptone 10 g; yeast extract 5 g; NaCl, 10 g.

Preparation of spheroplasts: Spheroplasts were prepared according to the procedure described earlier (Sadhu and Gopinathan 1982). The method consists of two steps: a) sensitization of the cells by exposure to glycine (0.2 M), and b) enzymatic degradation of cell wall material. Glycine was added to early log phase cultures of *M. smegmatis* and shaking was continued for 2 h at 37°C. The cells were harvested and resuspended in the original volume of 10 mM Tris (pH 7·0) containing 15% sucrose. Lipase and lysozyme were added to a final concentration of 500 μg/ml each and the incubation continued. Nearly 100% conversion of bacillary forms to spheroplasts was achieved within 2–3 h as monitored by phase contrast microscopy.

Fusion of spheroplasts: Spheroplast fusion was carried out by the following modification of the procedure for streptomyces described by Hopwood *et al* (1977). Equal amounts of spheroplasts of the strains to be fused were mixed and centrifuged at 5000 × g for 10 min. The pellet was resuspended in 0·1 ml of PEG-1000 and 0·14 ml of the minimal medium (without Tween-80) containing DMSO (15%). After 1 min, 0·1 ml PEG and 0·3 ml medium were added, which was followed after 3 min by the addition of 1·2 ml of medium. Samples of this suspension were layered on regeneration plates and incubated at 37°C for 3–4 days. Recombinants were screened by serially transferring the individual colonies on LB-agar plates containing novobiocin (25 μg/ml) and/or kanamycin (100 μg/ml).

3. Results and discussion

Formation of spheroplasts of M. smegmatis SN2: The treatment of *M. smegmatis* cells presensitized by growth in presence of glycine, with a combination of lipase and lysozyme yielded spheroplasts (figure 1). The presence of lipase was most essential for the production of spheroplasts. Due to the presence of large amounts and multiple layers of lipopolysaccharides, mycobacterial cell walls are resistant to lysozyme alone, which is widely used to prepare spheroplasts from gram-positive bacteria. The yield of spheroplasts was > 95% efficient and was achieved within 2–3 h.

Spheroplasts were stable for several hours at room temperature. They were lysed by treatment with 0·1% sodium dodecyl sulfate (SDS), in contrast to the parental cells.

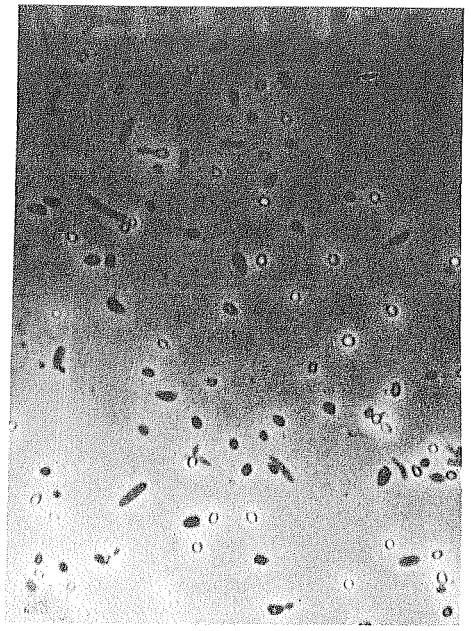
Regeneration and fusion of spheroplasts: The mycobacterial spheroplasts regenerated with fair efficiency on a suitable regeneration medium. They could be distinguished from the parental cells by separately scoring for the total regenerants and the osmotically resistant (after SDS treatment) cells plating on the regular medium. Thus, in a population of 5×10^8 cells, after spheroplasting $8{\cdot}5 \times 10^3$ cells remained resistant to SDS-lysis which correspond to the parental cell population. On the regeneration medium, however, $1{\cdot}8 \times 10^5$ colonies contributed both by the parental cells as well as the regenerated spheroplasts, were obtained. Therefore, 95% of the colonies on the regeneration medium arise from spheroplast regeneration.

Temperature is one of the important parameters which affects spheroplast regeneration (Hopwood 1981). To determine the optimum temperature of regeneration, *M. smegmatis* spheroplasts prepared by the above method were spread on regeneration medium and incubated at different temperatures. At 37°C, the regeneration of spheroplasts was better (1·5–2·0 times) than at the other temperatures tested.

Fusion of protoplasts has been reported in a large number of bacterial species but the frequencies vary considerably. Fodor and Alfoldi (1976) have reported a frequency of 10^{-5} to 5×10^{-3} in the case of *Bacillus megaterium* protoplasts. On the other hand, Frehel *et al* (1979) have observed a fusion frequency as high as 65% of the input protoplasts in *B. subtilis*. To check whether effective fusion is occurring under our conditions we have fused wild-type *M. smegmatis* spheroplasts with the spheroplasts prepared from *M. smegmatis* lysogenized with its temperate phage 13 and monitored prophage activation by plating the fused spheroplasts in the presence of indicator bacteria. In the plates which received the fused spheroplasts, the number of plaques were 25–30 times greater than that of the control plates which received the spheroplasts of the lysogen only, indicating effective fusion.

Generation of recombinant by spheroplast fusion: Spheroplasts were produced from novobiocin- and kanamycin-resistant cells of *M. smegmatis* and the spheroplasts from these two strains were fused with PEG. Recombinants were screened among the regenerated bacilli. The flow chart of the screening procedure is given in figure 2. The number of colonies appearing at each step is given in parentheses. As indicated, no recombinant appeared when the fused spheroplasts were directly plated on the regeneration medium containing both novobiocin and kanamycin. However, 836 colonies appearing on nonselective medium (plates having no drug) when individually transferred to drug containing plates, only 3–4% of them grew on either of the plates, contrary to expectation. In fact, the entire population should have grown on plates containing either novobiocin or kanamycin due to the parental phenotypes. This observation suggested that during the process of spheroplast formation or fusion, there was a loss of drug resistance properties in most of the cells.

The colonies which had grown on novobiocin were transferred to medium containing kanamycin or novobiocin, or both. All of them grew on novobiocin, some (3 out of 14) grew on kanamycin, and only one (out of 14) grew on medium



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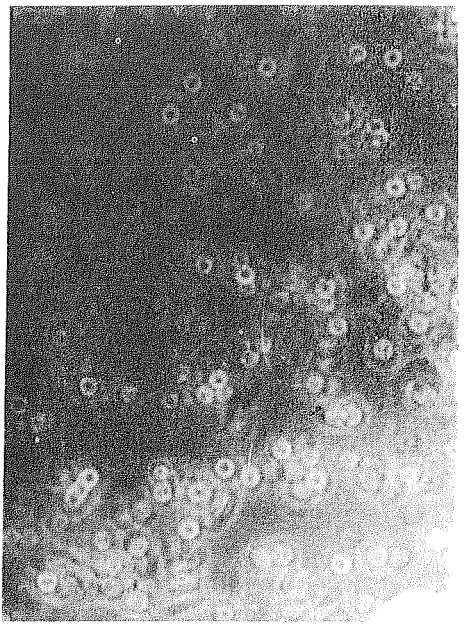

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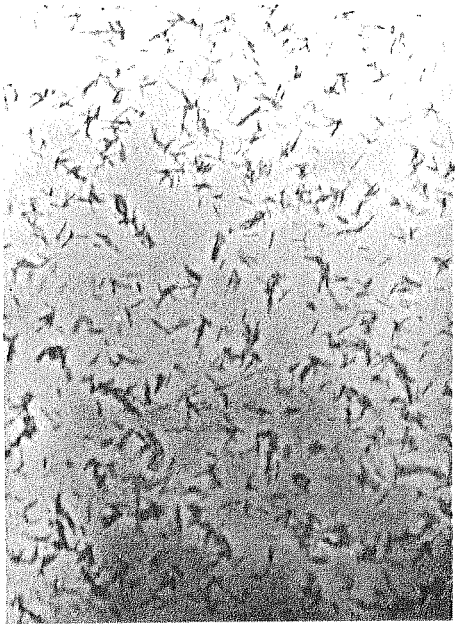

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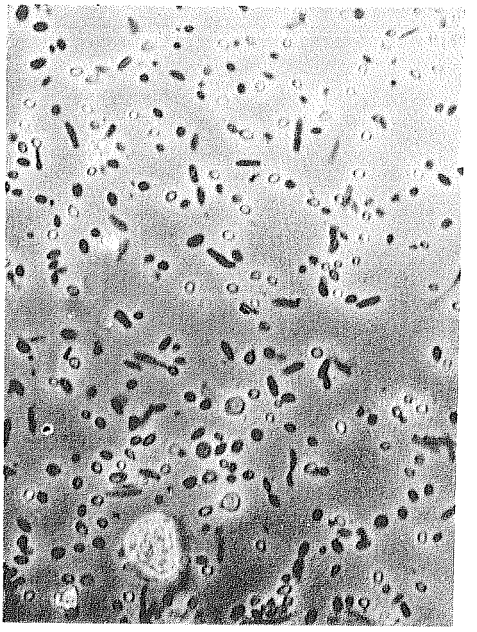

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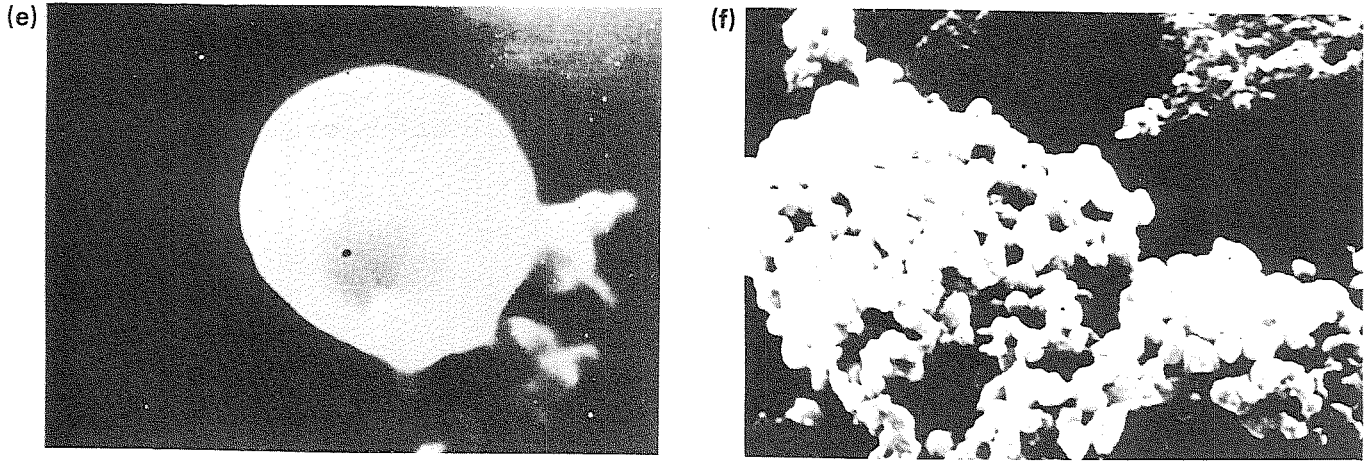

Figure 1. Spheroplasts of *M. smegmatis*. Bacterial cells grown in presence of 0·2 M glycine (presensitization) were treated with a combination of lipase and lysozyme (500 μg/ml each) as described in §2. Samples were examined under phase contrast microscope or scanning electron microscope. **(a)** Bacterial cells prior to enzymatic digestion; **(b–d)** cells treated with the enzymes for 1, 2 and 3 h respectively; **(e–f)** a single spheroplast or a clump of spheroplasts as seen under scanning electron microscope.

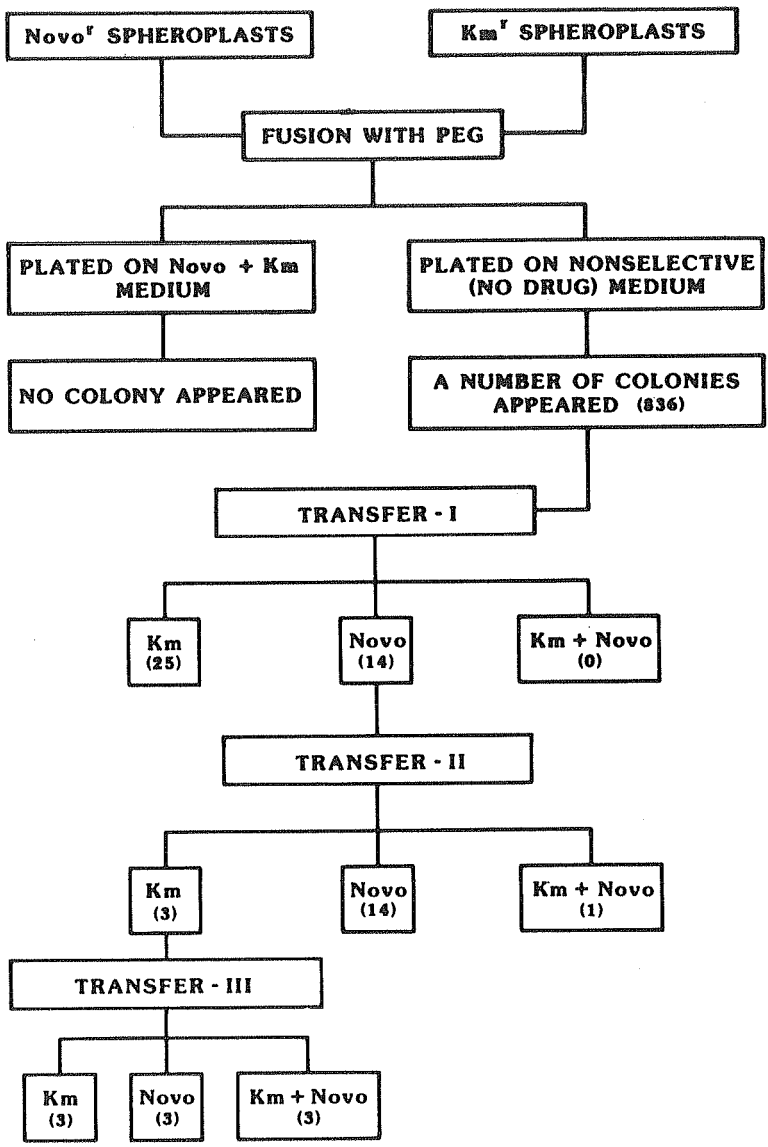

Figure 2. Flow chart for screening recombinants.

containing both novobiocin and kanamycin. Evidently, the loss of antibiotic resistance noted previously was only transient because resistance to kanamycin reappeared. Resistance to both the drugs, however, was found only in one colony. Subsequent transfer of the colonies from kanamycin plates on to medium containing kanamycin or novobiocin showed that all were now resistant to both antibiotics. After this period, the colonies appeared stable in terms of their antibiotic resistance and further loss or gain of resistance was not noted. A similar pattern was obtained for the colonies which were grown on kanamycin containing plates after transferring from the nonselective plates (Transfer-I).

These observations suggested that there was a lag in the expression of recombinant characters in the fused spheroplasts of *M. smegmatis*. In *B. megaterium* a transitory segregation of markers was observed after fusion of auxotrophic protoplasts (Fodor and Alfoldi 1976). Subsequently, Hotchkiss and Gabor (1983) have discussed different features of chromosome interaction and expression after fusion of *Bacillus* protoplasts and predicted the occurrence of 'chromosome inactivation' in the fused product. In our case, most of the clones resulting from spheroplast formation, fusion and regeneration initially expressed neither of the antibiotic resistances of the parents. This can be explained by a possible repression of one genome by the other before recombination as seen in the *Bacillus* system. On subsequent replica plating both the characters were found to be simultaneously and stably expressed. This may represent true recombinants.

In conclusion, we have demonstrated that (a) drug resistance markers can be introduced into *M. smegmatis* cells via spheroplast fusion, and (b) there is a considerable lag between the fusion step and appearance of recombinants. However, to make full use of the spheroplast fusion system for gene mapping in mycobacteria, further studies on the events subsequent to fusion are required.

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