

## Spheroplast Fusion as a Mode of Genetic Recombination in Mycobacteria

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Spheroplasts were prepared from two carotenoid pigment mutants of *Mycobacterium aurum* named NgR<sub>9</sub> and A<sub>11</sub>, which were obtained by the chemical mutagenesis of the wild type strain A<sup>+</sup> with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. The carotenoid pigments and the  $\alpha$ - and  $\beta$ -mycolic acids were taken as genetic markers and the recombinants were selected on the basis of their colour on Löwenstein–Jensen medium. Spheroplasts of the two mutants were mixed in a 1:1 ratio and were treated with 40% (w/v) polyethylene glycol 6000 for 5 min at 37 °C. The frequency of NgR<sub>9</sub> × A<sub>11</sub> recombination in optimal conditions was about  $2.5 \times 10^{-3}$ . The recombinants selected on the basis of their carotenoid pigment profile were also tested for their  $\alpha$ - and  $\beta$ -mycolic acids as a second genetic marker. The results were further confirmed by electron microscopy. The optimal conditions for spheroplast fusion as a mode of genetic recombination in *M. aurum* are described.

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### INTRODUCTION

Very few reports on genetic transfer in mycobacteria have been published; the topic has been reviewed in detail by Grange (1975, 1982) and Šlosárek *et al.* (1978). According to these reports, optimal conditions under which genetic transfer would be reliable and reproducible have not yet been found. Limited success in genetic transfers has been achieved by conjugation in *Mycobacterium smegmatis* (Mizuguchi & Tokunaga, 1971; Tokunaga *et al.*, 1973; Mizuguchi, 1974; Mizuguchi *et al.*, 1976). The only other mycobacterial species for which recombination has been attempted are *Mycobacterium phlei* and *Mycobacterium tuberculosis*, but mating strains were not found and attempts to induce mating competence in *M. phlei* by transferring fertility (F) plasmids from *Escherichia coli* were not successful (Koniček & Koničková-Radochová, 1975).

Transformation (Nordgard & Imaeda, 1978) and transfection (Nakamura, 1970) have been reported but success has been very limited in this field, probably due to a lack of suitable recipient strains or to the complexity of the mycobacterial cell wall. Better results by transduction have been claimed (Redmond, 1970; Gelbart & Juhasz, 1970; Sundar Raj & Ramakrishnan, 1970; Jones & David, 1972; Saroja & Gopinathan, 1973). However, the transducing system of Gelbart & Juhasz (1970) showed that up to 20% of the transfer activity was due to co-transfer of DNA. Because of the limited number of genes transferred, transduction can only be used for studying the fine structure of parts of bacterial genome, and would be unsuitable for the mapping of the bacterial genome.

Thus the genus *Mycobacterium* has played a very limited role in basic studies on molecular biology and genetics. According to Redmond (1970), the knowledge of genetics in mycobacteria in 1970 had reached only the stage that had been achieved in 1947 with *E. coli*. Because we had

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Abbreviations: LJ medium, Löwenstein–Jensen medium; PEG, polyethylene glycol.

already developed a method for spheroplast preparation from *Mycobacterium aurum* (Rastogi & David, 1981), we looked at fusion as a mode of genetic recombination in mycobacteria.

Fusion as a tool of genetic recombination was first reported for plant protoplasts (Power *et al.*, 1970; Carlson *et al.*, 1972), followed by fungal protoplasts (Ferenczy *et al.*, 1975; Anné & Peberdy, 1976), *Bacillus subtilis* (Schaeffer *et al.*, 1976; Gabor & Hotchkiss, 1979), *Bacillus megaterium* (Fodor & Alföldi, 1976; Fodor *et al.*, 1978), different *Streptomyces* species (Hopwood *et al.*, 1977; Hopwood & Wright, 1978, 1979; Baltz, 1978; Godfrey *et al.*, 1978; Ochi *et al.*, 1979; Gumpert, 1980) and yeasts (van Solingen & van der Plaats, 1977; Svoboda, 1978). The mechanism of fusion was recently studied electron microscopically by Frehel *et al.* (1979), and Gumpert (1980) has recently proposed a model for membrane alterations during fusion at the molecular level.

The above reports showed that (a) genetic recombination by protoplast fusion permitted transfer of larger genome parts than did conventional methods, or transfer of entire genomes of two or more cells; (b) genetic recombination at high frequency was possible even in bacteria for which conventional methods would not work; (c) stable recombinants could be isolated after fusion, once wall regeneration was achieved. In this paper, we show that fusion of mycobacterial spheroplasts results in genetic recombination and describe the optimal conditions required for fusion in *M. aurum*.

#### METHODS

*Organism and growth.* *Mycobacterium aurum* A<sup>+</sup> (wild-type, yellow coloured) was from our own culture collection. Cells maintained on slants of Löwenstein-Jensen (LJ) medium were used. Two carotenoid pigment mutants, A<sub>11</sub> (a first-stage mutant, white, devoid of carotenoid pigments) and NgR<sub>0</sub> (a brick red, second-stage mutant derived from A<sub>11</sub>) were obtained by chemical mutagenesis of the original A<sup>+</sup> strain (Lévy-Frèbault & David, 1979). Bacteria were grown as a shake culture in nutrient broth (Difco) at 37 °C. For carotenoid pigment studies, the bacteria were incubated for 12 d at 37 °C. For all other studies, the cells were harvested after 3 to 4 d.

*Spheroplast preparation and regeneration.* Spheroplasts were prepared as reported earlier (Rastogi & David, 1981). For spheroplast regeneration, appropriate dilutions of the spheroplast suspensions in Tris/HCl buffer (50 mM, pH 7.0) containing 20% (w/v) sucrose were plated on LJ medium in Petri dishes and the colonies were counted after one week, once the counts were stabilized. The rate of spheroplast regeneration under our experimental conditions was about 10<sup>-1</sup> for mutant NgR<sub>0</sub> and about 1.7 × 10<sup>-1</sup> for mutant A<sub>11</sub>.

*Spheroplast fusion.* The NgR<sub>0</sub> and A<sub>11</sub> spheroplasts were mixed in a 1:1 ratio (approximately 10<sup>8</sup> to 10<sup>9</sup> spheroplasts capable of regeneration; rate of spheroplast reconversion being about 10<sup>-1</sup> to 2 × 10<sup>-1</sup>). They were treated with DNAase type 1 (Sigma) (5 µg ml<sup>-1</sup>) in the presence of 20% (w/v) sucrose for 30 min at 37 °C, before treatment with polyethylene glycol (PEG). The two spheroplast types were sedimented together by centrifugation at 10000 g at room temperature, and 1 to 2 ml of a prewarmed (37 °C) solution of 40% (w/v) PEG 6000 in 50 mM Tris/HCl (pH 7.0) was added to the pellet followed by gentle mixing. At this stage, aggregation of spheroplasts could be detected by phase-contrast microscopy in a thin layer of 2% (w/v) agar. After incubation for 5 min (unless otherwise indicated) at 37 °C, the spheroplast suspension was diluted 10-fold in Tris/HCl buffer (50 mM, pH 7.0) containing 20% (w/v) sucrose and 5 µg DNAase type 1 ml<sup>-1</sup>. Centrifugation at this step was not necessary as PEG at 4% (w/v) had no effect on spheroplast fusion. All serial dilutions were performed in the same buffer and appropriate dilutions were plated on LJ medium for recombinant selection. Ca<sup>2+</sup> in concentrations from 10 to 100 mM has often been used in PEG-induced fusion experiments (Ochi *et al.*, 1979; Gumpert, 1980); however, its presence was not necessary in fusion experiments with *M. aurum*.

*Extraction and identification of carotenoid pigments.* The harvested cells, suspended in a minimum volume of water, were extracted several times with chloroform/methanol (1:1, v/v). An equal volume of petroleum ether (b.p. 40 to 60 °C) was added to the extracts, the mixtures were vigorously stirred with a Vortex mixer, and the pigments were then allowed to partition. The epiphasic pigments were then purified by preparative TLC on aluminium strips precoated with silica gel F<sub>254</sub> (Merck). The solvent used was 2% (v/v) acetone in *n*-hexane. The pigments were tentatively identified by comparing their absorption spectra and chromatographic properties with published data (Goodwin, 1954; Davies, 1965; Lévy-Frèbault & David, 1979). The carotenoid content of the extracts was estimated at 450 nm (Liaaen-Jensen & Jensen, 1971). To compare the R<sub>F</sub> values of different pigments, TLC was performed on silica gel G precoated plates (Merck).

*Extraction and identification of fatty acids.* The bacteria were grown in Middlebrook 7H9 medium (Difco), supplemented with 0.5% (w/v) Casitone (Difco) and 1% (w/v) glucose. The medium was dispensed in 250 ml Erlenmeyer flasks (100 ml per flask). The cultures were incubated at 37 °C with agitation for one week and the cells were harvested by centrifugation at the end of their exponential phase of growth.

Bacterial lipids were saponified by refluxing for 12 h in a mixture of ethanol/benzene/water (5:1:1, by vol.) containing 5% (w/v) KOH. After acidification, the mixture was washed with distilled water and was then concentrated under vacuum. The fatty acids were then methylated with diazomethane. The methyl esters were chromatographed on activated silica gel G plates (Merck), with a mixture of hexane/ether (8:2, v/v) as the solvent. The fatty acids were detected by spraying with a solution of 0.01% rhodamine B in 0.25 M-sodium phosphate. The definitive identification was made by co-chromatography with authentic standards.

The mycolic acids were characterized by mass spectroscopy on a Varian-Mat 311 A instrument. The electron energy was 70 eV, the source temperature was 250 °C and the direct probe temperature was about 280 °C. The methods used were the same as those described earlier (Promé *et al.*, 1976; Asselineau & Asselineau, 1978; Asselineau *et al.*, 1981).

*Electron microscopy.* Spheroplasts were prefixed for 2 h at 4 °C with 1% (w/v) glutaraldehyde (Sigma) in 0.2 M-cacodylate buffer (pH 6) containing 0.34 M-sucrose and 10 mM-Mg<sup>2+</sup>. After centrifugation, the pellets were resuspended in 2.5% (w/v) agar in nutrient broth. Small blocks were cut out and were fixed overnight (18 h) in 2.5% (w/v) glutaraldehyde in Michaelis-veronal buffer, washed twice with the same buffer, and postfixed with 1% (w/v) OsO<sub>4</sub> and then with 1% (w/v) uranyl acetate as reported earlier (Ryter & Kellenberger, 1958). Dehydration was performed with acetone and embedding with Epon (Ladd Research Laboratories, U.S.A.). Sections 40 nm thick were cut with a diamond knife on a Sorval microtome, stained with lead citrate and observed in a Siemens Elmiskop 101 electron microscope.

The position of fusing cytoplasmic membranes was located by silver proteinate coloration, as applied earlier to mycobacteria (Rastogi *et al.*, 1981, 1982).

## RESULTS

### *Recombinant selection and identification*

The character used for the preliminary selection of recombinants was the colour of the colonies formed on LJ medium. Mutants NgR<sub>9</sub> and A<sub>11</sub> formed brick red and white colonies respectively (the wild-type parental A<sup>+</sup> strain was orange-yellow). To establish if recombination between NgR<sub>9</sub> and A<sub>11</sub> mutants had taken place, mixtures of the respective spheroplasts (after PEG treatment, see below) were plated together and screened for yellow colonies. On plates we observed yellow-salmon coloured colonies with a frequency of  $2.2 \times 10^{-3}$ , and some sectorial colonies with a frequency of about  $5 \times 10^{-5}$  (Table 1, Fig. 1). Yellow-salmon coloured colonies were cloned.

The results of TLC of isolated carotenoid pigments, and their absorption spectra, and also the analysis of mycolic acids, confirmed that the recombinants were different from the parental strains (Table 1), as they formed carotenoid pigments not found in the parental strains, and as the distribution of mycolic acids in the recombinants was also distinct from the parental strains.

TLC of the total fatty acid methyl esters of the wild-type A<sup>+</sup> strain revealed five spots: mycosanoate ( $R_f$  0.77), esters of common fatty acids ( $R_f$  0.72),  $\alpha$ -mycolate ( $R_f$  0.44),  $\beta$ -mycolate

Table 1. *Recombination following fusion of NgR<sub>9</sub> × A<sub>11</sub> spheroplasts: distribution of mycolic acids and carotenoid pigments in M. aurum A<sup>+</sup> (wild type), NgR<sub>9</sub> and A<sub>11</sub> mutants and three randomly chosen recombinants* \*

Organism	Colour of colonies	Carotenoid pigments						Mycolic acids	
		Leptotene	$\alpha$ -Carotene	Lycopene	Neurosporene	Chlorobactene?	$\delta$ -Carotene	$\alpha$	$\beta$
A <sup>+</sup>	Orange-yellow	+	+	+	+	±	±	+	+
NgR <sub>9</sub>	Brick red	—	—	+	+	—	+	+	—
A <sub>11</sub>	White	—	—	—	—	—	—	+	+
R2	Yellow-salmon	—	+	+	+	—	+	+	+
R20	Yellow-salmon	—	—	+	+	+	—	+	+
R27	Yellow-salmon	—	+	+	+	—	—	+	+

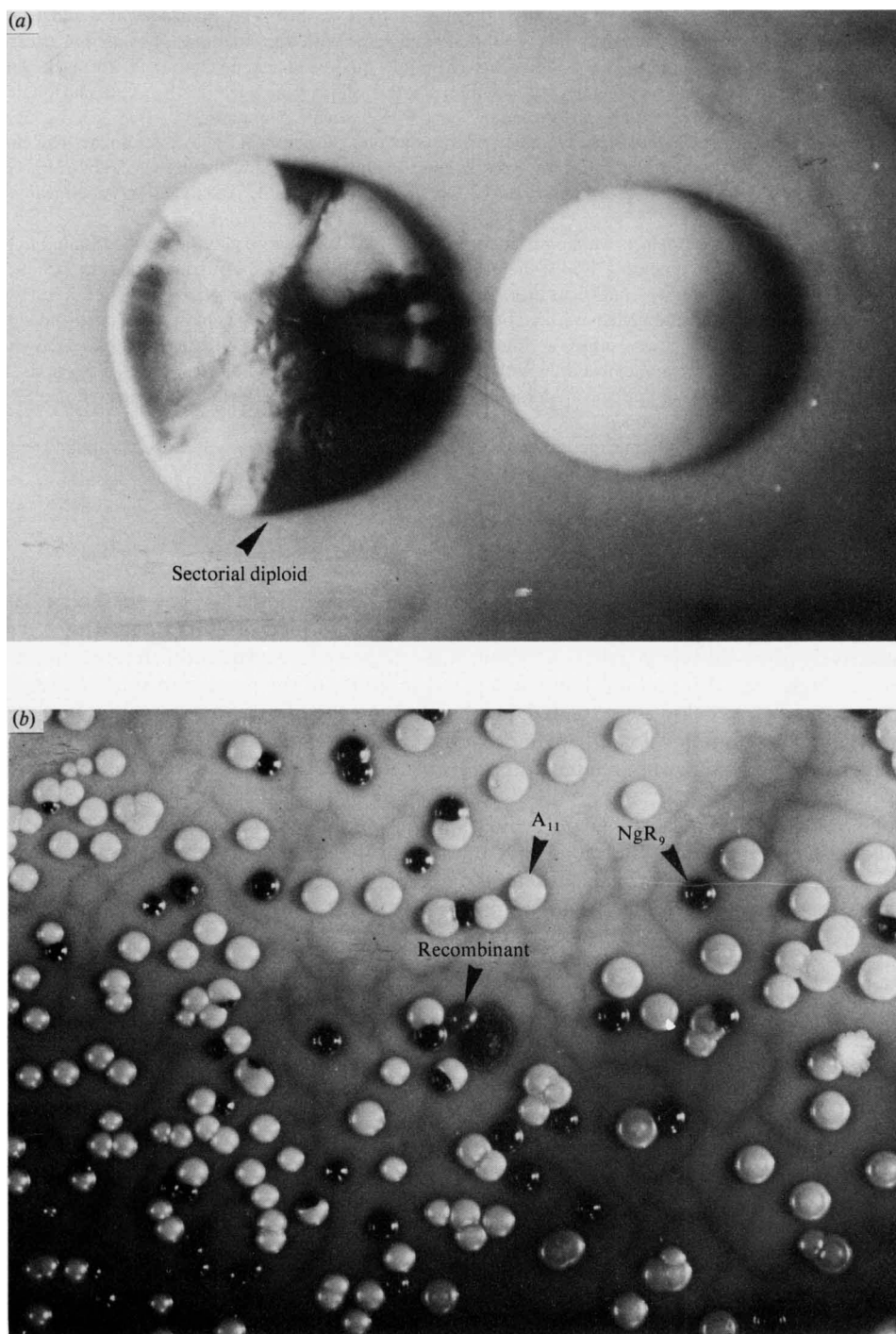


Fig. 1. Preliminary selection of diploid cells and recombinants after platings on LJ medium of PEG-treated NgR<sub>9</sub> × A<sub>11</sub> spheroplasts: (a) unstable diploids were found with a frequency of about  $5 \times 10^{-5}$ , and could be recognized by their typical sectorial colony phenotype; (b) yellow-salmon coloured recombinants were found with a frequency of about  $2.2$  to  $3.5 \times 10^{-3}$ .

( $R_F$  0.33), dicarboxylic mycolate ( $R_F$  0.17) and methyl carbinol ( $R_F$  0.17). TLC of total fatty acid methyl esters of the mutants NgR<sub>9</sub> and A<sub>11</sub> showed that while A<sub>11</sub> had the same profile as A<sup>+</sup>, NgR<sub>9</sub> was deficient in  $\beta$ -mycolate. The possibility that the  $\beta$ -mycolate could have been masked by  $\gamma$ -mycolate (i.e.  $\omega$ -methylcarbinol esters of dicarboxylic mycolates) was eliminated by verifying that  $\gamma$ -mycolates were fully hydrolysed in our experimental conditions.

The recombinants obtained were analysed for their fatty acid content. They all contained  $\beta$ -mycolates as did strains A<sup>+</sup> and A<sub>11</sub>, in addition to other fatty acids. These results showed that some form of genetic exchange took place during *M. aurum* spheroplast fusion; however, a true wild-type phenotype among the recombinant clones could not be found (Table 1). In previous studies on reversion rates of the individual mutants NgR<sub>9</sub> and A<sub>11</sub> (Lévy-Frébault & David, 1979; V. Lévy-Frébault, personal communication), as well as during our own control experiments, no revertants were found, possibly because each strain was a double or triple mutant. This observation is important in more than one aspect: firstly the yellow-salmon coloured colonies observed at frequencies greater than  $10^{-3}$  were not revertants of the individual mutants but a result of genetic exchange, and secondly it would also explain the absence of recombinant clones with a wild-type phenotype. Since fusions of more than two cells can occur, presumably resulting in various ploidy levels, the results are difficult to interpret at the genetic level.

In addition to the yellow-salmon coloured recombinants, we also observed a few sectorial colonies (Fig. 1, Table 2). When the sectorial colonies were resuspended in RVB 10 medium (Difco) and were successively transferred in the same medium and plated on LJ medium, we observed that the frequency of the sectorial colony types decreased rapidly in the colony population from  $6 \times 10^{-2}$  to  $8 \times 10^{-2}$  to  $10^{-5}$  or even less after three transfers (Fig. 2). These data showed that the sectorial colonies were developed from diploid or polyploid organisms. However, the diploidy or polyploidy appeared unstable as it segregated rapidly after a few transfers on a proper growth medium. It would be logical to expect that such sectorial colonies would generate recombinant clones on subculture, even if at a low frequency. However, in our own studies we did not search for these as it would have required a large number of dilution platings. Instead, we attempted to verify whether the diploidy or polyploidy in the above case was stable.

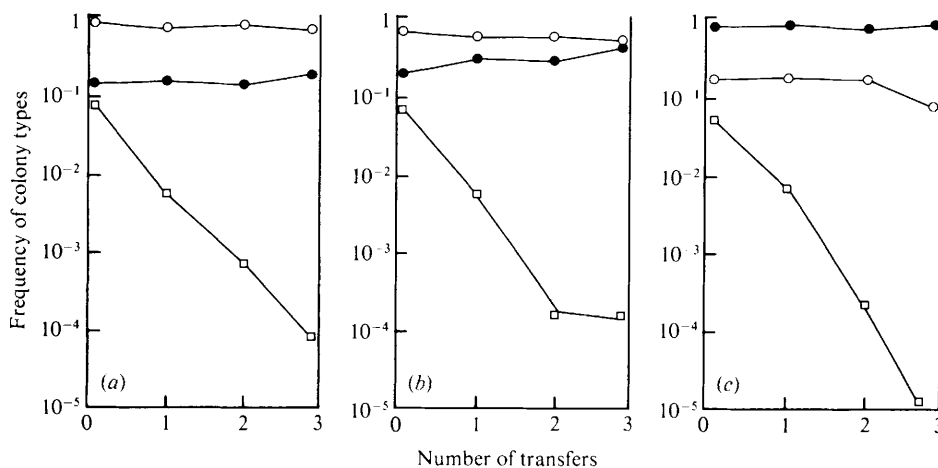


Fig. 2. Segregation of diploid bacteria (sectorial colonies) after successive transfers in RVB 10 medium: (a), (b) and (c) show the segregation of the sectorial colonies 2, 3 and 4 respectively (see Table 2). During each transfer about  $10^8$  initial bacteria were grown to give about  $10^{10}$  c.f.u. 0 on the transfer scale gives the frequency of different colony types among the initial diploid colonies. The data shown on this scale were obtained by direct platings of the above colonies on LJ medium (see Table 2). ●, NgR<sub>9</sub> cells; ○, A<sub>11</sub> cells; □, diploid cells.

Table 2. *Segregation of diploid bacteria (sectorial colonies)*

Sectorial colony 1 is shown in Fig. 1 (a); sectorial colonies 2, 3 and 4 were obtained from the platings of original colony 1 shown in Fig. 1 (a) and their segregation was studied after successive transfers in RVB 10 medium (Fig. 2). The results show the number of colonies with different phenotypes after direct plating of the indicated sectorial colonies on LJ medium.

Sectorial colony	Phenotype of colony population		
	Red (NgR <sub>9</sub> )	White (A <sub>11</sub> )	Sectorial
1	700	500	48
2	18	142	14
3	50	165	16
4	256	50	17

According to these data, spheroplast fusion in *M. aurum* resulted in genetic exchanges. Some of the resulting fusion products were transient diploids or polyploids, while in other cases, stable recombinants could be isolated. However, none of the recombinants isolated had a true wild-type phenotype.

#### *Effect of PEG-treatment on spheroplast regeneration and fusion*

The effects of PEG treatment were observed under different experimental conditions to find optimal conditions for *M. aurum* spheroplast regeneration and fusion. In all these experiments, the number of spheroplasts regenerated without any treatment was taken as 100% and all other values are expressed as the percentage of the spheroplasts regenerated. The fusion frequencies are calculated taking into account only the regenerated spheroplasts. The conditions tested were the following: (a) effect of different molecular weights of PEG, ranging from 200 to 20000 (Fig. 3); (b) effect of different concentrations of PEG-6000 (Fig. 4); (c) effect of the duration of treatment with 40% (w/v) PEG-6000 (Fig. 5); (d) effect of pH ranging from 3 to 9, during treatment with or without 40% (w/v) PEG-6000 for 5 min at 37 °C (Fig. 6).

(a) All PEG solutions of molecular weights ranging from 200 to 20000 were prepared at 40% (w/v) in distilled water (except PEG-20000 which was used only at 30% due to its high viscosity). The time of PEG treatment was 5 min at 37 °C. As shown in Fig. 3, the fusion was optimal with PEG-6000, which resulted in up to  $2.2 \times 10^{-3}$  recombinants. Increasing the molecular weight of PEG caused clumping of spheroplasts and decreased the number of regenerated spheroplasts.

(b) Figure 4 shows the effect of PEG-6000 in concentrations ranging from 0 to 60% (w/v) on spheroplast regeneration and recombinant frequency. The time of PEG treatment was 5 min at 37 °C. Though recombinant frequency was as high as  $3.5 \times 10^{-3}$  with 50% PEG-6000, there was high killing among spheroplasts with concentrations above 40%.

(c) When spheroplasts were treated with 40% (w/v) PEG-6000 at 37 °C for times ranging from 5 to 60 min (Fig. 5), the recombination frequency increased from  $4 \times 10^{-4}$  at time 0 (measured immediately after PEG addition) to about  $2.2 \times 10^{-3}$  within 5 min of treatment. Longer treatments did not significantly increase the recombination frequency but caused a significant decrease of the total regenerated spheroplasts.

(d) Figure 6 shows the effect of pH ranging from 3 to 9 during treatment with 40% (w/v) PEG-6000 for 5 min at 37 °C. For this experiment the PEG solution was prepared either in 50 mM-citrate buffer (pH range from 3 to 6) or in 50 mM-Tris/HCl buffer (pH range from 7 to 9) (all pH values at 20 °C). The serial dilutions were done in similar pH conditions and were then plated on LJ medium. The results obtained indicate that *M. aurum* spheroplasts were more tolerant of alkaline than acidic conditions. However, both the spheroplast regeneration and recombination frequency were maximal at pH 6.

#### *Ultrastructural observations*

Figures 7 and 8 show the ultrastructure of NgR<sub>9</sub> × A<sub>11</sub> spheroplasts after PEG treatment. An agglutination of the spheroplasts could be observed (Fig. 7). As up to  $3 \times 10^{-2}$  spheroplasts

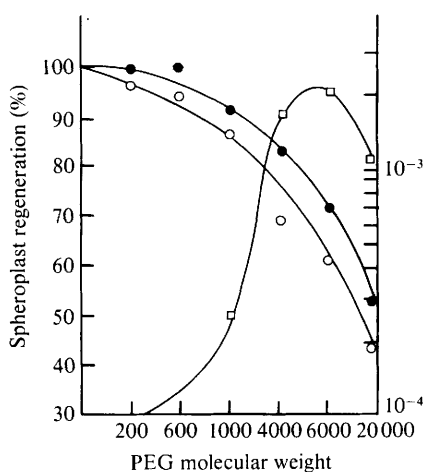


Fig. 3

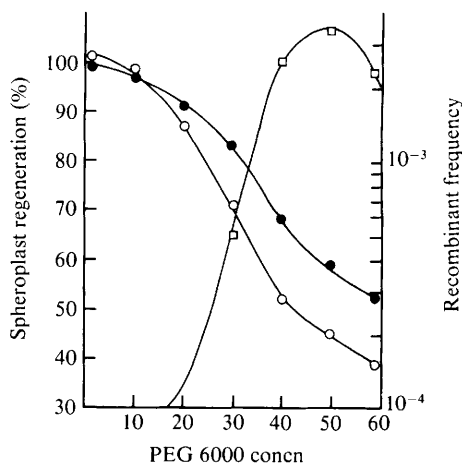


Fig. 4

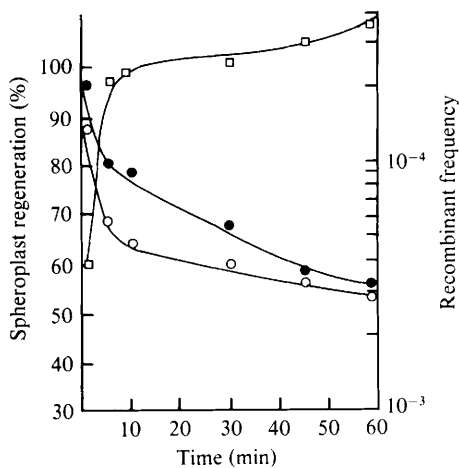


Fig. 5

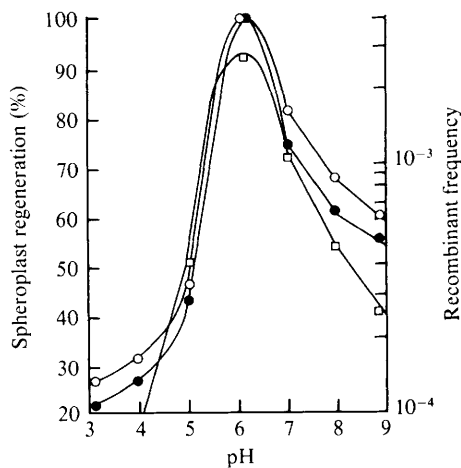


Fig. 6

Fig. 3. Effect of different molecular weights of PEG on spheroplast regeneration and recombination frequency. PEG of molecular weights ranging from 200 to 20000 was used at 40% (w/v); treatment was for 5 min at 37 °C.  $\bullet$ ,  $NgR_0$  spheroplast regeneration;  $\circ$ ,  $A_{11}$  spheroplast regeneration;  $\square$ , recombinant frequency.

Fig. 4. Effect of different concentrations of PEG-6000 on spheroplast regeneration and recombinant frequency. PEG-6000 was used in concentrations from 10% (w/v) to 60% (w/v); treatment was for 5 min at 37 °C.  $\bullet$ ,  $NgR_0$  spheroplast regeneration;  $\circ$ ,  $A_{11}$  spheroplast regeneration;  $\square$ , recombinant frequency.

Fig. 5. Effect of the duration of PEG treatment on spheroplast regeneration and recombination frequency.  $NgR_0 \times A_{11}$  spheroplasts were treated with PEG-6000 for different times ranging from 0 to 60 min at 37 °C.  $\bullet$ ,  $NgR_0$  spheroplast regeneration;  $\circ$ ,  $A_{11}$  spheroplast regeneration;  $\square$ , recombinant frequency.

Fig. 6. Effect of pH during PEG treatment of  $NgR_0 \times A_{11}$  spheroplasts. PEG solutions (40%, w/v) were prepared either in 50 mM-citrate buffer (pH range from 3 to 6) or in 50 mM-Tris/HCl (pH range from 7 to 9). Spheroplasts were treated for 5 min at 37 °C.  $\bullet$ ,  $NgR_0$  spheroplast regeneration;  $\circ$ ,  $A_{11}$  spheroplast regeneration;  $\square$ , recombinant frequency.

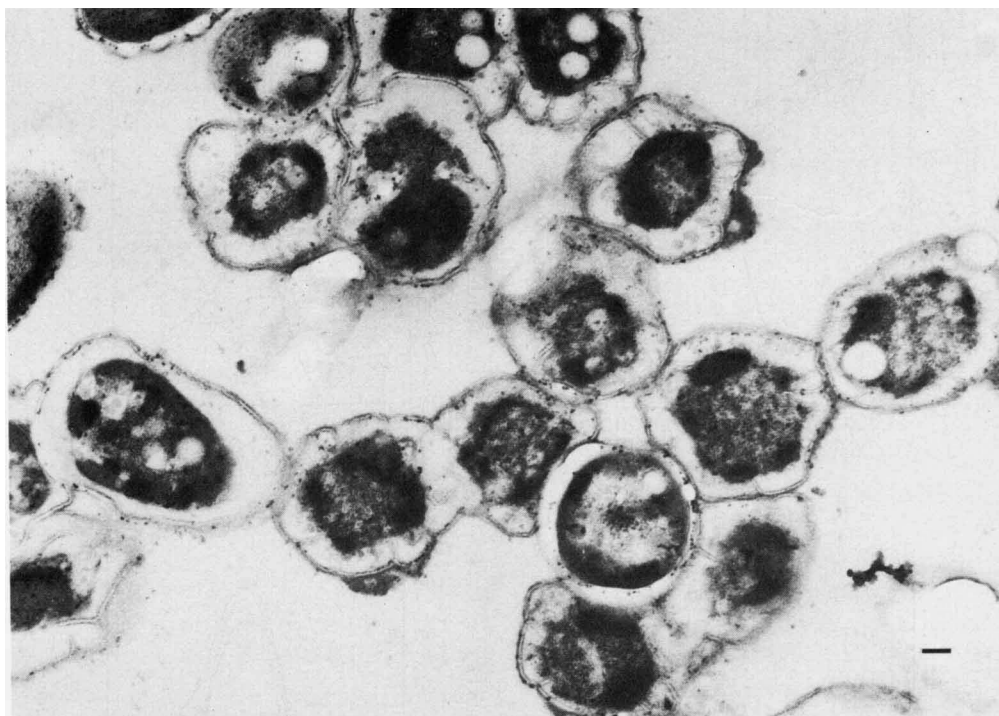


Fig. 7. Ultrastructure of PEG-treated  $\text{NgR}_9 \times \text{A}_{11}$  spheroplasts: after the addition of 40% (w/v) PEG-6000 to the spheroplast mixture, an immediate clumping could be observed. The bar marker represents 100 nm.

showed fusion events in electron microscopy compared to about  $2.2 \times 10^{-3}$  recombinants isolated, it appeared that only about one fusion event in 10 was able to give the desired recombinant.

Figures 8 (a), (b) and (c) show fusing spheroplasts at different stages; Fig. 8 (a) shows two spheroplasts sticking to each other with a contact zone still maintained. Sometimes a contact zone was associated with the formation of vacuoles (Fig. 8b). Figure 8(c) shows two perfectly fused spheroplasts, and only such fusion events should be considered as representative of the fusion event. Figure 8(d) might account for relatively rare events of fusion between more than two spheroplasts and would probably result in polyploidy. Similar multiple fusion events have been reported earlier from *Bacillus subtilis* (Frehel *et al.*, 1979). Figures 8(e), (f), (g) and (h) show different stages of spheroplast fusion at the contact zone level, ranging from a simple clumping (Fig. 8e) to complete fusion (Fig. 8h). The examples in Figs 8(f) and (g) are probably intermediate stages between the start and the end of the fusion process.

#### DISCUSSION

Our data show that when spheroplasts of two carotenoid pigment mutants of *M. aurum*  $\text{A}^+$  were mixed together in the presence of 40% (w/v) PEG-6000, recombinants could be isolated with a frequency of  $2.2 \times 10^{-3}$ . Though we did not find recombinants with PEG-200 and PEG-600, this was due to a lower recombination frequency rather than to the complete absence of recombinants. This was confirmed by the fact that in our control experiments without PEG treatment, fusion could be produced at very low frequencies (less than  $10^{-5}$ ) by high-speed centrifugation alone of the  $\text{NgR}_9 \times \text{A}_{11}$  spheroplasts at pH 6. This certainly was not a transformation event as DNAase type 1 ( $5 \mu\text{g ml}^{-1}$ ) was present during the centrifugation and

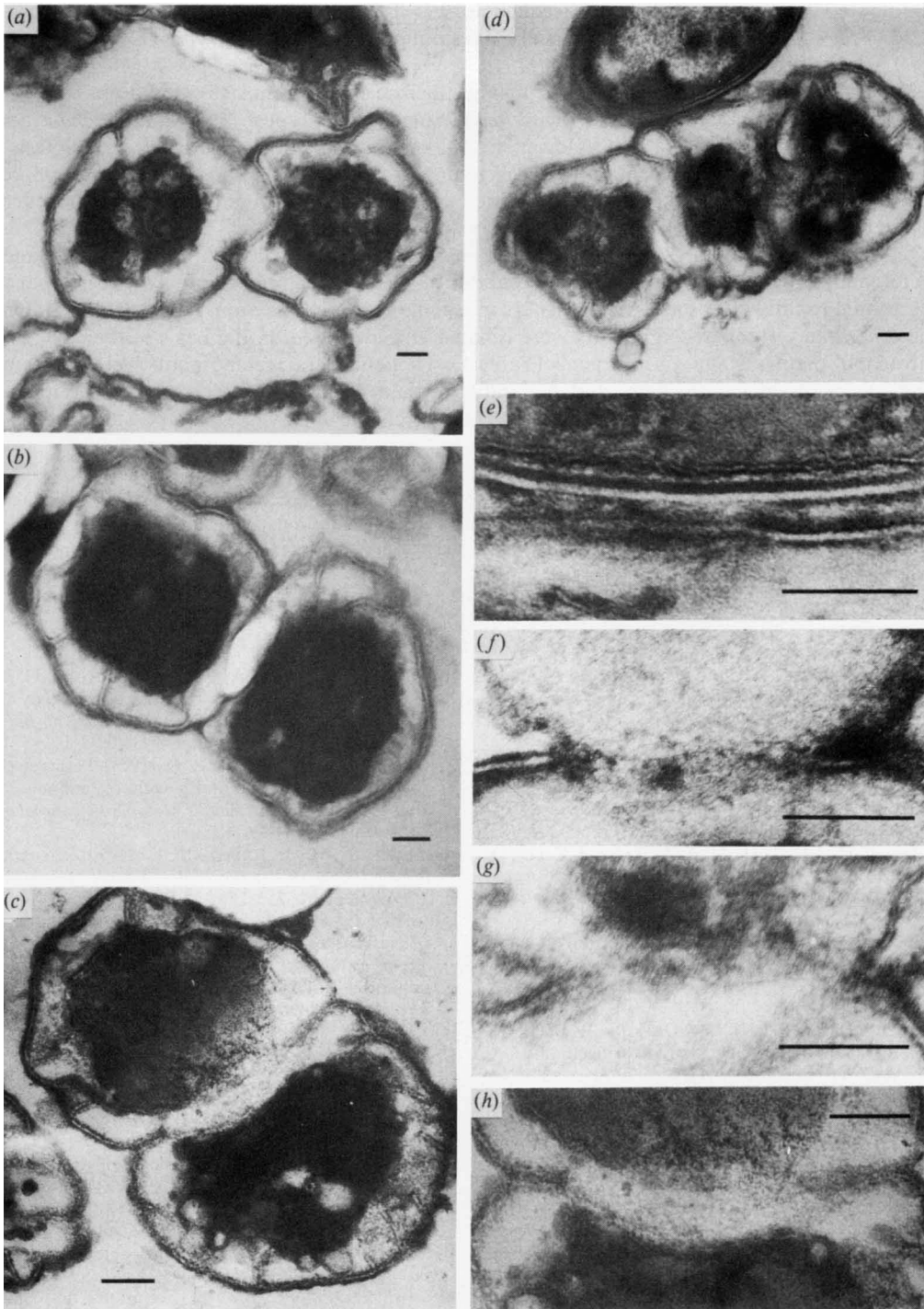


Fig. 8. Ultrastructure of fusing spheroplasts at different stages: (a) two spheroplasts clumping to each other with a well-maintained contact zone; (b) sometimes vacuoles could be observed at the contact zone level; (c) two spheroplasts which seem to be perfectly fused to each other; (d) a possible multiple fusion event; (e-h) different stages of fusion at the contact zone level, ranging from simple clumping (e) to complete fusion (h). The bar markers represent 100 nm.

plating steps. Optimal recombination frequencies were found when the spheroplasts were mixed in a 1:1 ratio and centrifuged together, and the resulting pellet was treated with 40% (w/v) PEG-6000 at pH 6 for 5 min at 37 °C.

The ultrastructural examination of the fusing spheroplasts gave images compatible with our data as well as with those found earlier with *Bacillus subtilis* (Frehel *et al.*, 1979) and *Streptomyces hygroscopicus* (Gumpert, 1980). However, unlike *Bacillus* or *Streptomyces* protoplasts, mycobacterial spheroplasts contain few wall remnants. This observation suggests that unlike *Escherichia coli* spheroplasts (Lévy, 1978), which do not yield recombinants due to the presence of the outer layer, mycobacterial wall remnants do not inhibit fusion.

Spheroplast fusion in *M. aurum* resulted in yellow-salmon coloured recombinants and sectorised diploids. The diploids were apparent due to a typical sectorial colony type (Fig. 1) and segregated rapidly after successive transfers in a suitable growth medium (Fig 2, Table 2). On the other hand, stable recombinants were isolated and identified on the basis of their colour, carotenoid pigments and mycolic acids (Table 1). Though all the recombinants were different from the parental strains NgR<sub>9</sub> and A<sub>11</sub>, none of them formed leprotene, one of the major pigments formed by the wild-type A<sup>+</sup> strain (Lévy-Frébault & David, 1979), possibly because NgR<sub>9</sub> and A<sub>11</sub> were double or triple mutants.

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