Genetic Recombination by Spheroplast Fusion of Sterol-transforming *Mycobacterium* Strains

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Wall-deficient forms of fast-growing mycobacteria were produced in growth medium containing vancomycin and glycine, and spheroplasts were prepared by lysozyme treatment of wall-deficient cells. Spheroplasts gave rise to recombinants with high frequency (2–6%) when they were fused using polyethylene glycol 6000. The results demonstrated that *in vivo* genetic recombination could be used to produce genetically modified *Mycobacterium* strains with applications in transformation of steroids. Useful intermediates of steroid drug synthesis and new degradation products were obtained from sterols by selected recombinant strains.

INTRODUCTION

Mycobacteria are widely used in microbiological transformation of steroids (Schubert *et al.*, 1961; Wix *et al.*, 1968; Ambrus & Büki, 1969) and hydrocarbons (Habets-Crutzen *et al.*, 1985). Classical mutagenic techniques have been successfully applied to strain improvement of steroid-transforming mycobacteria, resulting in industrially important strains which degrade naturally occurring sterols to produce intermediates for the synthesis of steroid drugs (Marsheck *et al.*, 1972; Wovcha *et al.*, 1978).

Limited or no success has been achieved in gene transfer by conjugation in different *Mycobacterium* spp. (Mizuguchi & Tokunaga, 1971; Koniček & Koničková-Radochová, 1975). However, the spheroplast fusion technique offers an alternative for studying the genetics of steroid-metabolizing *Mycobacterium* strains. Several methods have been published for the preparation of spheroplasts from *M. tuberculosis* (Thacore & Willett, 1963; Dorozhkova & Volk, 1972), from *M. bovis* BCG (Sato *et al.*, 1965) and from different fast-growing mycobacteria (Adámek *et al.*, 1969; Rastogi & David, 1981; Rastogi *et al.*, 1983*a*). Genetic recombinations have been carried out between carotenoid pigment mutants of *M. aurum* by spheroplast fusion (Rastogi *et al.*, 1983*b*, 1986).

In this paper we describe experiments on the preparation and fusion of spheroplasts of auxotrophic and antibiotic-resistant mutants of fast-growing *Mycobacterium* strains with sterol-degrading properties. The sterol-transforming properties of parent and recombinant strains are compared.

METHODS

Strains and growth conditions. *Mycobacterium* sp. NCAIM 00349 and *Mycobacterium phlei* NCAIM 00029 are deposited in the National Collection of Agricultural and Industrial Micro-organisms (Budapest). Cells were maintained on slants of glucose/potato medium, which contained 1% (w/v) glucose, 3% (w/v) potato starch and 2.2% (w/v) agar.

Auxotrophic and antibiotic-resistant mutants of the *Mycobacterium* strains were obtained by MNNG mutagenesis. The bacteria were inoculated into nutrient broth (6 g l⁻¹, Difco) supplemented with Tween 80 (0.1%), and incubated overnight at 37 °C with shaking. The cells were harvested by centrifugation, resuspended in 0.9% saline solution supplemented with 0.1% Tween 80, and incubated at 32 °C for 80 min with 500–2000 µg

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) ml⁻¹. After incubation, the suspension was spread onto glucose/potato agar plates and incubated at 32 °C for 5–7 d; isolated colonies were tested for nutritional requirements. By mutagenic treatment of *Mycobacterium* sp. NCAIM 00349 we obtained auxotrophic strains, which require for growth adenine (50 µg ml⁻¹), valine (50 µg ml⁻¹) and proline (50 µg ml⁻¹), respectively, and streptomycin-resistant mutants able to grow on agar media containing 500 µg ml⁻¹ streptomycin.

Spheroplast preparation and fusion. For preparation of wall-deficient forms the bacteria were grown at 32 °C for 48 h in shake flasks containing nutrient broth (6 g l⁻¹, Difco), then 1% of the precultured bacteria were inoculated and incubated for 16 h at 32 °C in nutrient broth medium containing glycine 1.5% (w/v). Finally, a membrane-sterilized solution of vancomycin (100 µg ml⁻¹ final concentration) was added to the resulting culture and incubation was continued for a further 3 h at 37 °C. The wall-deficient forms obtained from 100 ml cultures were harvested by centrifugation, washed three times with P buffer (Okanishi *et al.*, 1974; Hopwood *et al.*, 1985; but with 0.5 M-sucrose), resuspended in 4 ml P buffer containing lysozyme (0.5–10 mg ml⁻¹) and incubated at 32 °C for 180 min with continuous stirring. During this step, the decrease in turbidity of the bacterial suspension was monitored by measuring optical density at 620 nm. After lysozyme treatment, the suspension was centrifuged at 700 *g* for 1 min at 20 °C to remove cell debris, and the supernatant carefully decanted and centrifuged at 8000 *g* for 20 min at 20 °C to collect the spheroplast pellets. Fusion was promoted by treating spheroplast pellets for 5 min with 1 ml 50% (w/v) polyethylene glycol (PEG 6000, Fluka AG) in P buffer, followed by dilution with 4 ml buffer P and plating on a non-selective agar medium (Hopwood *et al.*, 1985). The basal regeneration medium contained sucrose (171 g), K₂SO₄ (0.25 g), MgCl₂ · 6H₂O (10.12 g), glucose (10 g), Casamino acids (0.1 g, Difco), Bacto-agar (22 g, Difco), and 800 ml distilled water. After autoclaving, the following solutions were added to 80 ml portions of the basal medium: 1 ml KH₂PO₄ (0.5%), 8 ml CaCl₂ · 2H₂O (3.68%), 1.5 ml L-proline (20%), 10 ml TES buffer (5.73%, adjusted to pH 7.2), 0.2 ml trace element solution, and 0.5 ml 1 M-NaOH. After the plates had been incubated for 5–7 d, the cells were re-suspended in distilled water and plated on a minimal medium containing glucose (10 g), NH₄NO₃ (1.0 g), K₂HPO₄ (0.25 g), MgSO₄ · 7H₂O (0.25 g), NaCl (0.005 g), FeSO₄ · 7H₂O (0.001 g), agar (15 g), distilled water to one litre, and combinations of growth factors selective for particular genotypes. Colonies were counted after 5 d incubation.

Electron microscopy. The wall-deficient forms and spheroplasts were fixed for 6 h in a solution containing 2% (w/v) formaldehyde and 1.25% (w/v) glutaraldehyde (Sigma) in KH₂PO₄/K₂HPO₄ buffer (100 mM, pH 7.1), followed by post-fixation in 2% (w/v) OsO₄, in 200 mM-KH₂PO₄/K₂HPO₄ buffer (pH 7.1), for 2 h. Ultrathin sections were prepared using an LKB IV ultramicrotome and stained according to Reynolds (1963).

Steroid-transforming properties of recombinant strains. Sterol transformations were carried out in 500 ml flat-bottomed flasks containing 100 ml culture medium. The culture medium contained glycerol (10 g), urea (0.5 g), soybean flour (2.0 g), CaCO₃ (3.0 g), NH₄Cl (3.0 g), KH₂PO₄ (0.5 g), and distilled water to one litre. A fine suspension of 20 g of a 2:1 mixture of β -sitosterol (I) and campesterol (II), 2 g Tween 80 and 6 g polypropylene glycol 2000 was dispersed by heating and added to 1 litre of the culture medium before autoclaving at 121 °C for 20 min. The sterile culture media were inoculated with the *Mycobacterium* cells and shaken on a rotary shaker (deflection 2.5 cm, 320 rotations min⁻¹) at 32 °C for 168 h. At the end of the experiment the culture broth was extracted exhaustively with ethyl acetate.

The extracts were analysed by thin-layer chromatography, with Kieselgel HF₂₅₄₋₃₆₆ (Merck) as adsorbent. The developing solvents were: A, ethyl acetate/n-heptane (7:3, v/v); B, acetone/benzene (1:1, v/v). Detection was in UV light, and by conc. H₂SO₄ treatment, for the presence of the mixture of β -sitosterol (I) and campesterol (II) and the following microbial degradation products of these sterols: 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III); 1,4-androstadiene-3,17-dione (IV); 9 α -hydroxy-4-androstene-3,17-dione (V); 9 α ,17 β -dihydroxy-4-androsten-3-one (VI); 9 α -hydroxy-3-oxo-23,24-dinor-4-chole-22-oic acid (VII) and its methyl ester (VIII); 9 α -hydroxy-3-oxo-23,24-dinor-4,17(20)-choladien-22-oic acid (IX); 9 α ,22-dihydroxy-23,24-dinor-4-chole-3-one (X); 9 α -hydroxy-3-oxo-23,24-dinor-4,17(20)-choladien-22-al (XI); 9 α -hydroxy-26,27-dinor-4-cholestene-3,24-dione (XII); 9 α -hydroxy-27-nor-4-cholestene-3,24-dione (XIII); mixture of 3-oxo-4-stigmasten-26-oic acid (XIV) and 3-oxo-(24R)-24-methyl-4-cholesten-26-oic acid (XV) and methyl esters of compounds XIV and XV (XVI and XVII); 9 α -hydroxy-3-oxo-4,24(25)-stigmastadien-26-oic acid (XVIII) and its methyl ester (XIX); 4-stigmasten-3-one (XX); (24R)-24-methyl-4-cholesten-3-one (XXI). Approximate *R_F* values were as follows. System A: I, 0.81; II, 0.81; III, 0.78; IV, 0.61; V, 0.4; VI, 0.3; VII, 0.1; VIII, 0.66; IX, 0.14; X, 0.35; XI, 0.5; XII, 0.62; XIII, 0.7; XIV, 0.75; XV, 0.75; XVI, 0.86; XVII, 0.86; XVIII, 0.47; XIX, 0.78; XX, 0.9; XXI, 0.9. System B: VII, 0.4; VIII, 0.79; IX, 0.5; X, 0.56; XI, 0.75.

The microbial degradation products were isolated from the culture broths of both fusion partners, regenerated single strains and some selected recombinant strains. Ethyl acetate extracts obtained from the cultures were evaporated *in vacuo*. The microbial transformation products were separated by column chromatography (adsorbent, silicic acid; eluent, ethyl acetate/n-hexane with gradually increasing ethyl acetate content) and by preparative TLC (adsorbent, Kieselgel HF₂₅₄₋₃₆₆; developing solvents as described above; elution of compounds with methanol).

The following compounds were identified by comparison with authentic standards (UV, IR, TLC, GLC): I, II, III, IV, V, XX and XXI. Compounds VI, VII, VIII, IX, X, XI, XII, XIII were identified by comparison with literature data (m.p., UV, IR, $^1\text{H-NMR}$, MS) described earlier (Wovcha *et al.*, 1978; Knight & Wovcha, 1980, 1984). The closely related transformation products XIV and XV were isolated as a mixture in which compound XIV is the main component as shown by mass spectrometry (XIV M^+ 442; XV M^+ 428). These compounds are described in the literature (Fujimoto *et al.*, 1982*a, b*). Compounds XVI and XVII were also obtained as a mixture which proved to be identical with the product of esterification of the mixture of XIV and XV carried out by diazomethane. The structures of compound XVIII and its methyl ester (XIX) were elucidated by UV, IR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and mass spectroscopy. *Compound XVIII*. UV: λ_{max} 239 nm, ($\epsilon = 21\,300\,1\text{ mol}^{-1}\text{ cm}^{-1}$) (overlapping absorption maxima of 4-en-3-one and 24-en-26-oic acid chromophores). MS: M^+ 456; $[\text{M}-\text{H}_2\text{O}]^+ = 438$ (base peak); $[\text{M}-\text{H}_2\text{O}-\text{CO}]^+ = 410$; $m/z = 124$ (ring A + C_6 fragment). *Compound XIX*. UV: λ_{max} 239 nm, ($\epsilon = 21\,500\,1\text{ mol}^{-1}\text{ cm}^{-1}$). MS: M^+ 470; $[\text{M}-\text{CH}_3\text{OH}]^+ = 438$; $[\text{M}-\text{CH}_3\text{OH}-\text{CO}]^+ = 410$; $m/z = 124$ (base peak). Structure determination of XVIII and XIX in detail will be published separately.

RESULTS

Preparation and regeneration of spheroplasts

The cell walls of the *Mycobacterium* strains studied could not be removed directly by lysozyme treatment. Pre-treatment with glycine and vancomycin, added to exponential-phase cultures, promoted the hydrolytic effect of lysozyme on the cell wall. Glycine and vancomycin each inhibited the growth of *Mycobacterium* sp. NCAIM 00349 (Fig. 1); the maximal inhibitory effect was observed when both glycine and vancomycin were added.

On the basis of this observation we have developed a suitable method for spheroplast preparation from fast-growing *Mycobacterium* strains. After incubation with glycine (1.5%, w/v) and vancomycin ($100\text{ }\mu\text{g ml}^{-1}$), the cells of *Mycobacterium* sp. NCAIM 00349 were digested with lysozyme (10 mg ml^{-1}). As shown in Fig. 2, some fragments of the cell wall remained attached to the membrane, hence most of the cells were converted to spheroplasts rather than true protoplasts. The enzymic hydrolysis resulted in conversion of approximately 85% of the cells into spheroplasts.

The optimal temperature of regeneration of spheroplasts of *Mycobacterium* sp. NCAIM 00349 was 32°C . The first visible colonies from regenerated spheroplasts appeared after 3–4 d incubation. The rate of spheroplast regeneration was about 8×10^{-1} .

The above method for spheroplast preparation was also found to be suitable for *Mycobacterium phlei* NCAIM 00029, using 0.2% (w/v) glycine, $100\text{ }\mu\text{g}$ vancomycin ml^{-1} , and $500\text{ }\mu\text{g}$ lysozyme ml^{-1} .

Spheroplast fusions

In order to perform spheroplast fusion experiments we used genetically marked strains of *Mycobacterium* sp. NCAIM 00349. We compared the frequencies of prototrophic recombinants obtained by treating *Mycobacterium* sp. NCAIM 00349 auxotrophic strains with PEG 1000 and PEG 6000 in concentrations ranging from 30 to 60% (w/v). When spheroplasts were treated with 50% PEG 1000 at 32°C for 5 min, the recombination frequency was 3.5×10^{-3} prototrophic recombinants per initial (unfused) spheroplast, whereas a frequency of 6.4×10^{-2} was obtained after treatment with 50% PEG 6000.

The fusion partners and crosses in two of the fusion experiments were the following. In the first experiment the Ade⁻Str^S mutant of *Mycobacterium* sp. NCAIM 00349 was fused with the Ade⁺Str^R mutant. In the second fusion experiment the Ade⁺Str^S and Ade⁻Str^R mutants were the parents. Analysis of colonies obtained by cell wall regeneration after the fusion experiments gave the following results. In the first experiment we examined 2500 regenerated colonies: 159 Ade⁺Str^S prototrophic recombinants and 11 Ade⁻Str^R double-marked recombinants were found. In the second experiment we obtained 34 Ade⁻Str^S and 23 Ade⁺Str^R recombinants from the analysis of 3700 regenerated spheroplasts.

Fig. 1. Lytic effect of vancomycin and glycine added to cultures of *Mycobacterium* sp. NCAIM 00349. ○, Control (no addition); ●, 1.5% glycine; □, 100 µg vancomycin ml⁻¹; ■, 100 µg vancomycin ml⁻¹ + 1.5% glycine.

Fig. 2. Electron micrograph of a thin section of a spheroplast obtained from *Mycobacterium* sp. NCAIM 00349. Bar, 0.1 µm.

Sterol-transforming properties of recombinant strains

We studied the sterol-transforming properties of 525 Ade⁺Str^S recombinant strains obtained in four crosses carried out between the mutants Ade⁻Str^S and Ade⁺Str^R of *Mycobacterium* sp. NCAIM 00349. The parent strains produced 9 α -hydroxy-4-androstene-3,17-dione (V) as a main product and mixture of compounds VII, VIII, IX, X and XI formed in smaller amount from

Fig. 3. Characteristic transformation products accumulated from phytosterols with *Mycobacterium* strains obtained by the spheroplast fusion technique.

commercially available sitosterol [2 : 1 mixture of β -sitosterol (I) and campesterol (II)]. Traces of compounds III and VI were also observed among the degradation products accumulated. The ratio of compound V to the total amount of the by-products was 3 : 1 for both parent strains. Five per cent of the recombinants showed significant changes in sitosterol-degrading ability compared to that of their parents. Most of the selected strains produced the same degradation products, but the proportions differed from those produced by the parent strains. Two recombinant strains did not accumulate 9 α -hydroxy-4-androstene-3,17-dione (V) at all and gave a mixture of dinorcholanoic acids (VII and IX) in high yield and compounds VIII, X and XI as minor components during transformation. One recombinant strain yielded transformation products XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX and XXI derived from the initial stages of the side-chain degradation pathway. Compounds XVIII and XIX are new sterol degradation products. Among the recombinants we found two strains which were not able to transform sitosterol at all. In some transformations carried out with regenerated spheroplasts we also observed changes in the ratio of conversion products when compared to non-spheroplasted strains.

DISCUSSION

Peptidoglycan biosynthesis in the bacterial cell wall consists of three stages: biosynthesis of uridine nucleotide precursors; utilization of these precursors to form peptidoglycan strands; and cross-linking of the linear strands. Each of these stages is specifically inhibited by certain antibiotics and amino acids. We have found that the antibiotic vancomycin, which inhibits the second stage of peptidoglycan biosynthesis (Siewert & Strominger, 1967), stimulates the

formation of wall-deficient forms of mycobacteria. Previously glycine, which is incorporated into the uridine nucleotide precursors in place of L-alanine (Strominger & Birge, 1965), has been widely used for preparation of wall-deficient forms of mycobacteria (Sato *et al.*, 1965; Adámek *et al.*, 1969; Rastogi & David, 1981). In our experiments, the concerted action of glycine and vancomycin afforded wall-deficient cells which could readily be attacked with lysozyme for hydrolysis of the glycoside bonds of the carbohydrate strands.

Genetic recombination by protoplast fusion in *Streptomyces* was described by Hopwood *et al.* (1977), and the technique developed by Baltz (1978). The methods elaborated for protoplast fusion in *Streptomyces* can clearly be utilized for fast-growing mycobacterial strains. Protoplasts obtained from *Streptomyces fradiae* and *Streptomyces griseofuscus* grown to the late exponential phase regenerate efficiently (Baltz, 1978); the protoplast regeneration and fusion efficiencies are strongly influenced by the incubation temperatures for cell growth and protoplast regeneration (Baltz & Matsushima, 1981). We have shown that spheroplasts can be prepared in high yield from mycobacteria grown to the late exponential phase. After optimization of temperature for cell growth and spheroplast regeneration we obtained genetic recombinations at frequencies of $2-6 \times 10^{-2}$, similar to the frequencies described in *Streptomyces* (Hopwood *et al.*, 1977).

After simple regeneration of spheroplasts we obtained some strains with modified sterol-transforming properties, but they occurred at much lower frequencies than when spheroplast fusion was promoted by adding polyethylene glycol. Among the recombinants obtained by the spheroplast fusion method we found a strain that accumulated new transformation products which could be fitted into the microbial degradation pathway of sitosterol elucidated by Sih and coworkers (Fujimoto *et al.*, 1982*a, b*). Our method could be applied to isolate strains which produce 9 α -hydroxy-4-androstene-3,17-dione (V), the known intermediate of corticosteroid synthesis (Dodson & Muir, 1961; Van Rheenen & Shephard, 1979), from phytosterols at higher yields than the parent strains and to select strains which transform β -sitosterol (I) and campesterol (II) into 9 α -hydroxy-3-oxo-23,24-dinor-4-cholesterol-22-oic acid (VII) and 9 α -hydroxy-3-oxo-23,24-dinor-4,17(20)-choladien-22-oic acid (IX), useful starting materials for synthesis of steroid drugs with a pregnane side chain (Kieslich, 1985) (see Fig. 3).

Consequently, spheroplast fusion seems to be a promising technique for strain improvement programmes for mycobacterial transformation of steroids.

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REFERENCES

- ADÁMEK, L., MIŠOŇ, P., MOHELSKÁ, H. & TRNKA, L. (1969). Ultrastructural organization of spheroplasts induced in *Mycobacterium sp. smegmatis* by lysozyme or glycine. *Archiv für Mikrobiologie* **69**, 227-236.
- AMBRUS, G. & BŰKI, K. G. (1969). Degradation of sapogenins by *Mycobacterium phlei*. *Steroids* **13**, 623-625.
- BALTZ, R. H. (1978). Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. *Journal of General Microbiology* **107**, 93-102.
- BALTZ, R. H. & MATSUSHIMA, P. (1981). Protoplast fusion in *Streptomyces*: conditions for efficient genetic recombination and cell regeneration. *Journal of General Microbiology* **127**, 137-146.
- DODSON, R. M. & MUIR, R. D. (1961). Microbiological transformations. VII. The hydroxylation of steroids at C-9. *Journal of the American Chemical Society* **83**, 4631-4635.
- DOROZHKOVA, I. R. & VOLK, A. V. (1972). Induction rate of L-form in *Mycobacterium tuberculosis* under the effect of cycloserine and its combinations with other drugs. *Antibiotiki* **17**, 838-844.
- FUJIMOTO, Y., CHEN, C.-S., SZELECZKY, Z., DI TULLIO D. & SIH, C. J. (1982*a*). Microbial degradation of the phytosterol side chain. 1. Enzymatic conversion of 3-oxo-24-ethylcholesterol-4-en-26-oic acid into 3-oxo-cholesterol-4-en-24-oic acid and androst-4-ene-3,17-dione. *Journal of the American Chemical Society* **104**, 4718-4720.
- FUJIMOTO, Y., CHEN, C.-S., GOPALAN, A. S. & SIH, C. J. (1982*b*). Microbial degradation of the phytosterol side chain. 2. Incorporation of $\text{NaH}^{14}\text{CO}_3$ onto the C-28 position. *Journal of the American Chemical Society* **104**, 4720-4722.
- HABETS-CRUTZEN, A. Q. H., CARLIER, S. J. N., DE BONT, J. A. M., WISTUBA, D., SCHURIG, V., HARTMANS, S. & TRAMPER, J. (1985). Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. *Enzyme and Microbial Technology* **7**, 17-21.
- HOPWOOD, D. A., BIBB, J. M., CHATER, K. F., KIESER, T., BRUTON, C. J., KIESER, M. M., LYDIATE, D. J., SMITH, C. P., WARD, J. M. & SCHREMPF, H. (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.

- HOPWOOD, D. A., WRIGHT, H. M., BIBB, M. J. & COHEN, S. N. (1977). Genetic recombination through protoplast fusion in *Streptomyces*. *Nature, London* **268**, 171–174.
- KIESLICH, K. (1985). Microbial side-chain degradation of sterols. *Journal of Basic Microbiology* **25**, 461–474.
- KNIGHT, J. C. & WOVCHA, M. G. (1980). Microbial degradation of the phytosterol side-chain to 24-oxo-products. *Steroids* **36**, 723–730.
- KNIGHT, J. C. & WOVCHA, M. G. (1984). Isolation and quantitation of 9-hydroxy-3-oxo-23,24-dinor-4,17(20)-cholesterol-22-al from a sitosterol bioconversion. In *Advances in Steroid Analysis '84*, pp. 479–484. Edited by S. Görög. Amsterdam: Elsevier.
- KONIČEK, J. & KONIČKOVÁ-RADOCHOVÁ, M. (1975). Possibilities of the conjugation process in mycobacteria. *Folia microbiologica* **20**, 382–388.
- MARSHECK, W. J., KRAYCHY, S. & MUIR, R. D. (1972). Microbial degradation of sterols. *Applied Microbiology* **23**, 72–77.
- MIZUGUCHI, Y. & TOKUNAGA, T. (1971). Recombination between *Mycobacterium smegmatis* strains Jucho and Lacticola. *Japanese Journal of Microbiology* **15**, 359–366.
- OKANISHI, M., SUZUKI, K. & UMEZAWA, H. (1974). Formation and reversion of streptomycete protoplasts: culture conditions and morphological study. *Journal of General Microbiology* **80**, 389–400.
- RASTOGI, N. & DAVID, H. L. (1981). Ultrastructural and chemical studies on wall-deficient forms, spheroplasts and membrane vesicles from *Mycobacterium aurum*. *Journal of General Microbiology* **124**, 71–79.
- RASTOGI, N., LÉVY-FRÉBAULT, V. & DAVID, H. L. (1983a). Spheroplast formation from nine rapidly-growing mycobacteria. *Current Microbiology* **9**, 201–204.
- RASTOGI, N., DAVID, H. L. & RAFIDINARIVO, E. (1983b). Spheroplast fusion as a mode of genetic recombination in mycobacteria. *Journal of General Microbiology* **129**, 1227–1237.
- RASTOGI, N., RAUZIER, J. Y., PAPA, F. P. & DAVID, H. L. (1986). Biochemical and cultural analysis of mycobacterial recombinants obtained by spheroplast fusion. *Annales de microbiologie* **137A**, 135–142.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **32**, 27.
- SATO, H., DIENA, B. B. & GREENBERG, L. (1965). Spheroplast induction and lysis of BCG strains by glycine and lysozyme. *Canadian Journal of Microbiology* **12**, 255–261.
- SCHUBERT, K., BÖHME, K.-H. & HÖRHDOLD, C. (1961). Bildung einer Katosäure durch mikrobiellen Abbau von Progesteron. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **325**, 260–262.
- SIEWERT, G. & STROMINGER, J. L. (1967). Bacitracin: an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 767–773.
- STROMINGER, L. & BIRGE, C. H. (1965). Nucleotide accumulation induced in *Staphylococcus aureus* by glycine. *Journal of Bacteriology* **89**, 1124–1127.
- THACORE, H. & WILLETT, H. P. (1963). Formation of spheroplasts of *Mycobacterium tuberculosis* by lysozyme treatment. *Proceedings of the Society for Experimental Biology and Medicine* **114**, 43–47.
- VAN RHEENEN, V. & SHEPHARD, K. P. (1979). New synthesis of corticosteroids from 17-keto steroids: application and stereochemical study of the unsaturated sulfoxide-sulfonate rearrangement. *Journal of Organic Chemistry* **44**, 1582–1584.
- WIX, GY., BÜKI, K. G., TÖMÖRKÉNY, E. & AMBRUS, G. (1968). Inhibition of steroid nucleus degradation in mycobacterial transformations. *Steroids* **11**, 401–413.
- WOVCHA, M. G., ANTOSZ, F. J., KNIGHT, J. C., KOMINEK, L. A. & PYKE, T. R. (1978). Bioconversion of sitosterol to useful steroidal intermediates by mutants of *Mycobacterium fortuitum*. *Biochimica et biophysica acta* **531**, 308–321.