THIRTY-FIRST SYMPOSIUM OF THE SOCIETY FOR GENERAL MICROBIOLOGY

GENETICS AS A TOOL IN MICROBIOLOGY

CAMBRIDGE

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EDITED BY

S. W. GLOVER AND D. A. HOPWOOD

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EDITORS' PREFACE

The last Society symposium devoted exclusively to the genetics of micro-organisms was held twenty-one years ago (Hayes & Clowes, 1960). It is not surprising, therefore, that that symposium concentrated, in large measure, on the elucidation of genetic mechanisms in micro-organisms since the previous decade 1950–60 witnessed many important milestones in bacterial genetics including the discovery of genetic transduction and conjugation in bacteria and the description of a new class of genetic elements – the episomes (Jacob, Schaeffer & Wollman, 1960).

Twenty-one years later our understanding of these phenomena is, in many respects, almost complete. It is entirely appropriate, therefore, that this symposium concentrates on the application of genetics as a tool, an indispensable tool, in the analysis of many fundamental aspects of microbiology.

Those who edit books and organise symposia are faced with the formidable task of selection. Our aim has been to choose topics of wide interest and fundamental importance in which the application of genetic methods has led to rapid progress and new insights in recent years. The reader will judge how far this symposium achieves that aim. The limitations of time and of printed space have inevitably led to the exclusion of many interesting topics and it is our hope that these may be included in some future symposium of our Society.

We thank all our authors who laboured long to produce manuscripts in time to meet our stringent deadlines and we thank Cambridge University Press for their help in the production of this volume.

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MICROBIAL PROTOPLAST FUSION

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INTRODUCTION

The field of induced fusion of microbial protoplasts (i.e. cells completely deprived of the wall) is a rapidly expanding one, and many data have accumulated in both basic and applied areas since the first reports on complementation of auxotrophic mutants by controlled protoplast fusion (Ferenczy, Zsolt & Kevei, 1972; Ferenczy, Kevei & Zsolt, 1974). Results of intra- and interspecific fusion obtained in the past few years clearly indicate the possibilities and importance of this new method of genetic transfer. Different aspects of microbial protoplast fusion have been dealt with in various recent reviews (Ferenczy, Kevei & Szegedi, 1976; Peberdy, 1978, 1979a, 1980a, b; Alföldi, 1980; Fodor, Rostás & Alföldi, 1980; Cocking, 1980; Ferenczy, 1980). The aim of this paper is to give a short review of the whole area of induced protoplast fusion, from bacteria to algae, and to discuss the various consequences of genetic transfer.

The first observations on bacterial protoplasts and their fusion stem from as early as 1925 (Mellon, 1925). It might be supposed that artefacts were seen in the stained preparation; however, subsequent observations on preparations stained similarly and also claiming to demonstrate fusion events (Smith, 1944) were followed by direct examination of living material by time-lapse photography, and confirmed the validity of the finding of fusion of 'large bodies' (Dienes & Smith, 1944). This observation on *Bacteroides* strains was later corroborated, also by time-lapse microscopic photography, on *Proteus vulgaris* (Stempen & Hutchinson, 1951) and on *Bacillus anthracis* protoplasts (Stähelin, 1954).

The discovery of the spontaneous protoplast fusion of bacteria was followed by that of fungi, on *Saccharomyces* and *Candida* species (Müller, 1966, 1970), *Polystictus versicolor* (Strunk, 1967) and *Fusarium culmorum* (Lopez-Belmonte, Garcia Acha & Villanueva, 1966).

These observations in that period of protoplast research had the

common features that: (i) the fusion took place mainly during protoplast formation; (ii) its frequency was rather low and incalculable; and (iii) both partners ('parents' of the fusion products) were wild-type, with identical genetic backgrounds.

It is interesting to note that Lederberg & St Clair (1958) gave an account of an unsuccessful experiment aiming at complementation by controlled protoplast fusion of genetically marked *Escherichia coli* strains. As they concluded: 'Attempts to detect the fusion of protoplasts of sexually incompatible (F^-) genotypes were unrewarding. The design of the experiments was similar to that for DNA-transduction, mixtures of protoplasts being evoked and grown together in penicillin agar or broth. We also tried graded osmotic shocks, and spinning a mixed protoplast suspension in 10% sucrose in an air turbine centrifuge of 80 000 *g* for 20 min. Whereas the pellet showed evidence of considerable lysis, there was no indication of fusion of protoplasts either from microscopy or tests for recombinants.' And: 'Further studies are needed to establish whether protoplasts stemming from different lines of cells can fuse with genetically interesting consequences.'

Fourteen years later the first controlled protoplast fusion and complementation had been achieved with the yeast-like filamentous fungus *Geotrichum candidum* (Ferenczy, Zsolt & Kevei, 1972). After another four years the first successful controlled intraspecific protoplast fusions were reported for bacteria (Fodor & Alföldi, 1976; Schaeffer, Cami & Hotchkiss, 1976) and yeast (Sipiczki & Ferenczy, 1976), as well as interspecific fusions of *Aspergillus* (Ferenczy, 1976) and *Penicillium* species (Anné, Eyssen & De Somer, 1976). The fusion of *Streptomyces* (Hopwood, Wright, Bibb & Cohen, 1977) and subsequently algal protoplasts (Matagne, Deltour & Ledoux, 1979) also proved successful.

BASIC METHODS OF PROTOPLAST FUSION

The principles and procedures whereby wall-deprived cells can be obtained by using lytic enzymes and/or inhibitors of cell wall synthesis in the presence of osmotic stabilizers, and whereby conditions can be created for the spherical protoplasts to revert to the normal, wall-bearing microbial form, are well established for both prokaryotic microbes (Weibull, 1953, 1958; Lederberg, 1956; McQuillen, 1960; Spizizen, 1962; Park, 1968; Kaback, 1971; Sagara

et al., 1971; Okanishi, Suzuki & Umezawa, 1974; Fodor, Hadlaczky & Alföldi, 1975; Hadlaczky, Fodor & Alföldi, 1976; Weiss, 1976; Marquis & Corner, 1976; Landman & De Castro-Costa, 1976; Peberdy, 1979b; Hopwood, Wright, Bibb & Ward, 1979) and eukaryotic ones (Nečas, 1956a, b, 1980; Eddy & Williamson, 1957; Villanueva, 1966; Villanueva & Garcia Acha, 1971; Ferenczy, Kevei & Szegedi, 1975b; Peberdy, 1976, 1979a; Ferenczy, Vallin & Maráz, 1977; Maráz & Ferenczy, 1979a).

In most cases, mutants with auxotrophic, antibiotic-resistance, temperature-sensitive, respiration-deficient, morphological and/or colour markers are used for fusion, and the resulting complementation is the indication of successful protoplast fusion. Of course, all the necessary controls have to be employed to detect reverse mutations or genetic transfer achieved in other ways than by protoplast fusion. The calculation of frequency of fusion is normally based upon the frequency of complementation, although these two events can never be exactly the same; for example fusion will also occur between non-complementary identical partners. The frequency of fusion is usually determined by comparing the number of complemented colonies (e.g. colonies growing on incomplete medium when auxotrophic partners are used) to that of the non-complemented ones (colonies growing only on complete medium). The presumed fusion products have to be characterized by using cytological, biochemical and genetic methods.

In early fusion experiments a centrifugal force (Ferenczy, Zsolt & Kevei, 1972; Ferenczy, Kevei & Zsolt, 1974; Binding & Weber, 1974) and/or intensive aggregation of protoplasts in cold KCl osmotic stabilizer (Ferenczy, Kevei & Szegedi, 1975a) were applied to induce their fusion. After the discovery that polyethylene glycol (PEG) acts as a fusogenic agent of plant protoplasts (Kao & Michayluk, 1974; Wallin, Glimelius & Eriksson, 1974), this compound was soon introduced into experiments with the aim of protoplast or cell fusion ranging from microbial protoplasts (Ferenczy, Kevei & Szegedi, 1975b, c; Anné & Peberdy, 1975a, b) to mammalian cells (Pontecorvo, 1975; Maggio, Ahkong & Lucy, 1976; Pontecorvo, Riddle & Hales, 1977; Gefter, Margulies & Scharff, 1977; Hales 1977; O'Malley & Davidson, 1977; Wacker & Kaul, 1977), and is nowadays used almost exclusively.

Interestingly, PEG has been utilized not only as a special dehydrating agent for enzyme precipitation (Foster, Dunnill & Lilly, 1973) or protein crystallization (McPherson, 1976), but at a lower

concentration, also to stabilize protoplasts (Weibull, 1953; Wallin & Ericksson, 1973), and to stimulate nuclear division and wall formation (Wallin & Eriksson, 1973).

The methodological studies on PEG-induced fungal protoplast fusion (Ferenczy, Kevei & Szegedi, 1975b, 1976; Anné & Peberdy, 1975b, 1976) revealed that PEG preparations with molecular weights of 4000 or 6000 (PEG 4000 and PEG 6000, respectively) are optimum in fusion induction in the concentration range 25–40% in the presence of 10–100 mM $CaCl_2$. The addition of Ca^{2+} ions is critical for the attainment of high-frequency fusion. Ions or molecules as additional osmotic stabilizers in the PEG solution yield lower efficiencies, and this phenomenon is concentration-dependent. In experiments on bacterial protoplast fusion, high molecular weight PEG (PEG 6000) was employed (Fodor & Alföldi, 1976; Schaeffer, Cami & Hotchkiss, 1976; Kaneko & Sakaguchi, 1979; Tsenin, Karimov & Ribchin, 1978; Coetzee, Sirgel & Lacatsas, 1979), while in experiments with *Streptomyces* or *Micromonospora*, PEG preparations with different molecular weights were applied, such as PEG 1000 (Hopwood & Wright, 1978, 1979; Hopwood, Wright, Bibb & Ward, 1979), PEG 1540 (Hopwood, Wright, Bibb & Cohen, 1977), PEG 4000 (Ochi, Hitchcock & Katz, 1979), and PEG 6000 (Baltz, 1978).

Though the molecular mechanism of fusion by the PEG-Ca^{2+} fusogenic system is not exactly known, more and more of its details have recently become evident. Most of this newer knowledge derives from biophysical and freeze-fracture electron microscopic studies on the PEG-induced fusion of erythrocytes and erythrocyte membranes. Since the basic composition and structure of the cell membrane involved in the fusion are fairly similar throughout the whole of the living world, it is reasonable to assume that the fusion processes are also similar. This belief is strongly corroborated by the fact that fusion between erythrocyte cells and yeast protoplasts can be attained easily and with comparatively high yield (Ahkong *et al.*, 1975).

The sequence of fusion events starts with agglutination of the protoplasts caused by intensive dehydration, and the formation of aggregates of various extents. The protoplasts shrink and become highly distorted. Large areas of adjacent protoplasts come into very close contact (Ferenczy *et al.*, 1976). The next possible event, observed in erythrocyte membranes, is the translocation of intramembrane protein particles at the sites of close contact and their

aggregation (Knutton, 1979; Knutton & Pasternak, 1979). The following step seems to be lipid–lipid interactions between the adjacent protein-denuded membranes. Perturbation and reorganization of the lipid molecules, strongly promoted by Ca^{2+} ions (Ito & Ohnishi, 1974; Papahadjopoulos, Poste, Schaeffer & Vail, 1974; Ahkong, Fischer, Tampson & Lucy, 1975; Lansman & Haynes, 1975; Papahadjopoulos, Vail, Pangborn & Poste, 1976; Cullis & Hope, 1978; Ingolia & Koshland, 1978; Sun, Hasang, Day & Ho, 1979), results in fusion in small regions of membranes in contact. Small cytoplasmic bridges are formed, which then enlarge, and the two protoplasts fuse.

Ever since the first observations on PEG-induced protoplast fusion (Kao & Michayluk, 1974), it has repeatedly been emphasized that at least partial removal of PEG from the suspension is needed to obtain high-frequency protoplast fusion. When microbial protoplasts are treated with PEG and the agglutinated protoplasts are then mixed with the osmotically-stabilized culture medium, the above-mentioned requirement for a high yield of fusion products is met. On the other hand, it has been found that a high frequency of nutritional complementation based upon protoplast fusion can be routinely obtained with amino acid-requiring auxotrophic mutants of *Aspergillus nidulans* in the fusogenic PEG-Ca^{2+} solution supplied with components of the minimal medium (L. Ferenczy, unpublished).

PROKARYOTIC PROTOPLAST FUSION

Protoplast fusion of Gram-positive bacteria

The protoplast fusion of prokaryotic organisms gives a unique opportunity for the bringing together of two (or more) complete genomes, instead of transferring fractions of DNA by transformation, transduction or conjugation.

Transfer of genetic information proved especially useful with *Bacillus megaterium*, the Gram-positive bacterium for which no genetic transfer mechanism had previously been known. Double auxotrophic mutants of *B. megaterium* were used by Fodor & Alföldi (1976) to obtain nutritionally complemented fusion products. Their progeny analysis showed the appearance of parental, stable recombinant and transitory segregating phenotypes. By fus-

ing polyauxotrophic *B. megaterium* protoplasts it was clearly demonstrated that the physiological effects of cultivation and those of the different culture media employed during regeneration strongly influenced the yield of recombinants and distorted the expression of the genetic system (Fodor & Alföldi, 1979). A somewhat similar observation was reported for *Bacillus subtilis* (Gabor & Hotchkiss, 1979), with the conclusion that the regeneration of recombinant-forming fused protoplasts is different from the average regeneration for the population. The use of a fusion system for genetic analysis of these bacteria, therefore, seems to be complicated.

Thermal inactivation of one of the partners provides an opportunity for protoplast fusion and the selection of the recombinant fusion products, even when one of the partners is prototrophic. *B. megaterium* protoplasts lose the ability to revert to the bacillary form if incubated at 50 °C for 120 min. On the other hand, the heat-treated protoplasts can contribute to the formation of recombinants when fused with a viable partner (Fodor, Demiri & Alföldi, 1978).

From the genetic consequences of protoplast fusion of auxotrophic mutants of another Gram-positive bacterium, *B. subtilis*, similar conclusions can be drawn as for *B. megaterium*. Schaeffer, Cami & Hotchkiss (1976) reported the nutritional complementation of polyauxotrophic *B. subtilis* strains by protoplast fusion. It was concluded that transient diploids were obtained and that the only stable fusion products were haploid recombinants. Unselected markers segregated among the selected recombinants. No auxotrophic bacteria were found as segregants from prototrophic fusion products growing in a non-selective medium. The frequency of prototroph formation depended on the number and the chromosomal location of the auxotrophic markers used. It has further been demonstrated that not only viable, but also streptomycin-killed protoplasts of *B. subtilis* can be used as partners in fusion with living protoplasts of a streptomycin-resistant strain to obtain recombinants (Levi, Sanchez-Rivas & Schaeffer, 1977). When the protoplasts originated from sporulating cells of *B. subtilis*, protoplasts with two or more enclosed prespores could be observed in very high frequencies by electron microscopy (Frehel, Lheritier, Sanchez-Rivas & Schaeffer, 1979). High-frequency protoplast fusion of *B. subtilis* was also revealed by a prophage complementation test (Sanchez-Rivas & Garro, 1979) in which two strains, each lysogenic for a different

Sus mutant of the phage φ105, were induced by mitomycin-C, protoplasted, fused with PEG, and plated with φ105-sensitive indicator bacteria.

It was recently discovered that several per cent of the fusion products derived from polyauxotrophic *B. subtilis* strains are biparental, containing the unchanged genomes of both partners. Interestingly, a substantial proportion can be cloned as biparental cells for many generations. Their phenotype during the 'diploid' phase is that of one or the other auxotrophic parental type, and is not prototrophic. It is assumed that the particular chromosome can be replicated, but not expressed (Hotchkiss & Gabor, 1980).

Brevibacterium flavum protoplasts were induced by penicillin treatment from cells of this industrially-important bacterium, for which no transformation, transduction or conjugation mechanism has ever been found. Strains with auxotrophic and antibiotic (streptomycin and rifampicin) resistance markers were employed in the fusion process and selection was made for double antibiotic resistance. Stable haploid recombinants were obtained (Kaneko & Sakaguchi, 1979).

Protoplast (sphaeroplast) fusion of Gram-negative bacteria

Up till now, only two cases of fusion and complementation of protoplasts (sphaeroplasts) of Gram-negative bacteria have been reported (Tsenin, Karimov & Ribchin, 1978; Coetzee, Siergel & Lecatsas, 1979). With these microbes a part of the cell wall may not be completely dissolved by lysozyme and the additive compounds, so that the cell membrane, at least partially, will be covered by this. For this reason, the term 'sphaeroplast' may be more appropriate if the complete removal of the cell wall is not proved.

Two polyauxotrophic F^- mutants of *Escherichia coli* were selected, protoplasted (electron microscopic investigation revealed the formation of true protoplasts), fused, tested for nutritional complementation, and analysed genetically (Tsenin, Karimov & Ribchin, 1978). The overwhelming majority of the primary prototrophic colonies were not stable and the segregation of markers continued over many generations. With regard to segregation patterns and the requirements of the resulting clones, these colonies could be classified into the following groups: (i) mixed colonies containing stable recombinants and parental auxotrophs; (ii) mixed

colonies with unstable prototrophs and a few unstable recombinants; and (iii) colonies uniformly composed of stable prototrophic cells.

The other Gram-negative bacterial species successfully used for sphaeroplast fusion was *Providencia alcalifaciens* (Coetzee, Siergel & Lecatsas, 1979). Though sphaeroplasts were produced, it was revealed by electron microscopy that about 15% of them showed breaks in the residual cell wall, these breaks exposing areas of underlying cytoplasmic membrane. The fusion-induced recombinants originating from the sphaeroplast mixture of the auxotrophic partners were haploids, as in the case of Gram-positive bacteria. Analysis of the prototrophic colonies revealed the presence of stable prototrophs, or mixtures of stable prototrophs and stable recombinants. Parental types were not found. Unselected markers segregated among the recombinants. The frequencies of recombination depended on the number of chromosomal loci used in selection.

Protoplast fusion in Actinomycetales

Streptomyces protoplast fusion achieved by Hopwood and coworkers (Hopwood, Wright, Bibb & Cohen, 1977; Hopwood & Wright, 1978, 1979; Hopwood, Wright, Bibb & Ward, 1979) opened up new possibilities of gaining more detailed knowledge on *Streptomyces* genetics, and also of making advances in the practical application of the protoplast fusion technique. At present, fusion of protoplasts seem to be far the most promising method for the strain improvement of *Streptomyces* and similar species known as producers of antibiotics and other important compounds.

With different *Streptomyces* species (*S. coelicolor, S. parvulus, S. lividans, S. griseus, S. acrymicini*), all the three major processes – formation, fusion and regeneration of protoplasts – can be carried out efficiently. Recombination frequencies are high: routinely above (sometimes much above) 1%. The frequency of recombinants in the progeny can be increased by adding dimethyl sulphoxide (14%, v/v) to the PEG solution and/or by UV irradiation of the protoplasts immediately before fusion (Hopwood & Wright, 1979). With *S. coelicolor*, recombinants can easily be obtained even under non-selective conditions, and these will constitute 10–20% of the total spore progeny of the regenerated cultures (Hopwood & Wright, 1978, 1979; Hopwood, Wright, Bibb & Ward, 1979). These

extremely favourable conditions have the advantage that selectable markers need not be used to obtain recombinants in good yield.

Analysis of the recombinants of multiple crossover classes (Hopwood & Wright, 1979) revealed that, in contrast to conjugation or other means of gene transfer, genomes of both partners brought together by protoplast fusion are complete or nearly so, resulting in a transient diploid or quasi-diploid state of the fusion products. The similarity to events in eubacteria is obvious. The heterozygous diploid state was transient in *S. coelicolor* and fusion colonies frequently contained only recombinants without parentals; in other cases both recombinant and parental genomes could be found. In the same colony, the presence of different recombinant genotypes was also observed. It was postulated that the genomes of both partners became fragmented after fusion. Crossing-over between the fragments gave rise to stable haploid recombinants. Events of recombination were independent of the known sex factors SCP1 and SCP2. Not only two-partner, but successful simultaneous three- and four-partner fusions, generating crossing-over and production of recombinants, has been reported (Hopwood & Wright, 1978).

Despite marked technical dissimilarities, the above observations of high-frequency recombination were confirmed by Baltz (1978), who studied the genetic consequences of protoplast fusion of auxotrophic and antibiotic resistant mutants of *Streptomyces fradiae*.

Highly-efficient intraspecific gene transfer and nutritional complementation was achieved in auxotrophic strains of *Streptomyces parvulus* and *Streptomyces antibioticus* (Ochi, Hitchcock & Katz, 1979). For recombination, the efficiency of protoplast fusion over the mating technique was 10^4 times higher. In *S. parvulus* stable prototrophic recombinants were obtained, while in *S. antibioticus* both stable prototrophs and nutritionally complemented unstable heterokaryons could be isolated, which is the typical form of labile complementation predominant in filamentous fungi and mycelial (pseudomycelial) yeasts (Ferenczy, 1980).

Rifampicin-resistant and casamino acid-dependent double mutants were produced from *Micromonospora echinospora* and *M. inyoensis* and fused with the corresponding antibiotic-sensitive wild-type parental strains by Szvoboda *et al.* (1980). By both direct and indirect methods, prototrophic and antibiotic-resistant colonies were selected. A direct selection method could be used effectively if the selecting antibiotic was added only after a phenotypic lag period