Genetic Recombination in Fused Spheroplasts of *Providencia alcalifaciens*

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Spheroplasts of *Providencia alcalifaciens* strain P29 auxotrophs were prepared by combined treatment with glycine and lysozyme–EDTA. About 15% of spheroplasts had areas of cytoplasmic membrane exposed where cell wall was absent. The spheroplasts of different auxotrophs were mixed pairwise and fusion was attempted with polyethylene glycol or nascent calcium phosphate. After spheroplasts had regenerated to bacterial forms selection was made for recombinants. Recombinants arose at frequencies of 3.8×10^{-6} to 1.7×10^{-7} per spheroplast initially present, by both methods of fusion. The frequency was strongly dependent on the number of chromosomal loci used in selection. The possible order of five loci was determined and this corresponded to that on the closely related *Proteus mirabilis* chromosome. Control experiments excluded possibilities of auxotrophic reversion, conjugation, transformation, transfection or transduction as explanations of the results. Analysis of prototrophic clones yielded stable prototrophs or mixtures of stable prototrophs and stable recombinants. Parental types were not encountered. Unselected markers segregated among recombinants. It was concluded that the formation of recombinant bacteria was due to spheroplast fusion and that only stable products of the very temporary heteroploid state were haploid recombinants. The low frequency of recombination was ascribed to the limited number of spheroplasts with areas of exposed cytoplasmic membrane.

INTRODUCTION

Fodor & Alföldi (1976) and Schaeffer *et al.* (1976) described the production of genetic recombinants by fusion of protoplasts of *Bacillus megaterium* and *Bacillus subtilis*, respectively. Hopwood *et al.* (1977), Hopwood & Wright (1978) and Baltz (1978) encountered frequent recombination accompanying protoplast fusion in *Streptomyces* spp.

There have been no reports regarding fusion of spheroplasts of Gram-negative bacteria. Lederberg & St Clair (1958) recorded their failure to detect fusion of penicillin-induced 'protoplasts' of *Escherichia coli*. Various F⁻ auxotrophic strains were used and criteria for positive results would have been the presence of clumping observed microscopically and ability to detect genetic recombination.

Van Rensburg (1969) demonstrated that lysozyme and EDTA treatment of a *Providencia alcalifaciens* strain converted more than 99% of cells to spheroplasts. Similar treatment of a strain of *Proteus mirabilis* converted only 80% of cells.

We decided to attempt spheroplast fusion in *Providencia alcalifaciens*, despite the fact that the chromosome of *Proteus mirabilis* strain PM5006 has been mapped (Coetzee, 1979*a, b*) and knowledge of marker location would have been useful (see Schaeffer *et al.*, 1976; Hopwood & Wright, 1978). There were three reasons for our choice. Firstly, in preliminary attempts to prepare spheroplasts by the combined method used (see Methods) the conversion frequency of *Providencia alcalifaciens* strain P29 to spheroplasts was higher

than that of *Proteus mirabilis* strain PM5006 and only spheroplasts of the former had areas of cytoplasmic membrane exposed. Secondly, *Proteus mirabilis* strain PM5006 is cryptically lysogenic for a generalized transducing phage (Krizzanovich, 1973) and this might have complicated the interpretation of fusion experiments. *Providencia alcalifaciens* strain P29, despite extensive searches, has not been proved lysogenic (unpublished, and see Coetzee, 1963). Thirdly, due to the ability of *Proteus mirabilis* to swarm and the soft agar method used to plate spheroplasts (see Coetzee, 1972; Williams & Schwarzhoff, 1978), difficulties in obtaining pure isolates of regenerated clones of bacteria were anticipated. *Providencia* spp. may also spread or 'emanate' in soft agar, but at 37 °C this tendency is absent in most strains (Coetzee & De Klerk, 1964).

METHODS

Bacteria. These are listed in Table 1.

Bacteriophage techniques. These were according to Adams (1956).

Media and solutions. Nutrient broth was Bacto-Penassay Broth code 0243-01. MacConkey agar was from Difco. Minimal medium (MM) was that of Grabow & Smit (1967). For auxotrophic strains, this medium was supplemented with amino acids, adenine or uracil (each at 30 µg ml⁻¹) when necessary. Rich regeneration medium (RRM) was nutrient broth containing 0.01 % (w/v) casein hydrolysate (Oxoid), 0.5 M-sucrose, 20 mg deoxyribonuclease I ml⁻¹ (Calbiochem) and 1.5 % (w/v) Bacto-agar (Difco). RRM top-layer agar was similar but contained only 0.6 % (w/v) Bacto-agar. MM, MacConkey agar and RRM contained 50 µg nalidixic acid ml⁻¹ to counter contamination. The lysozyme-EDTA spheroplasts were prepared in the sucrose/magnesium/maleate (SMM) buffer of Wyrick & Rogers (1973) to which 20 µg deoxyribonuclease I ml⁻¹ had been added (SMMD). The glycine treatment was done in medium G which was nutrient broth containing 0.5 M-sucrose and 4 % (w/v) glycine; after autoclaving, 0.01 M-MgCl₂ and 0.2 % (w/v) MgSO₄ · 7H₂O were added as Millipore-filtered solutions. Tris buffer was 0.067 M at pH 8, and the EDTA solution was 4 % (w/v); both solutions were autoclaved. Lysozyme (Sigma) was prepared in Tris buffer at 3 mg ml⁻¹. The polyethylene glycol (PEG) solution (Hopwood *et al.*, 1977) consisted of 40 % PEG (w/v) (mol. wt 6000) and 0.5 M-sucrose; after autoclaving a solution of dimethylsulphoxide was added to a concentration of 15 % (w/v). Incubation temperature was 37 °C.

Spheroplast preparation. A combined glycine and lysozyme-EDTA method was used. Bacteria grown overnight in 40 ml aerated nutrient broth culture (titre, 2 × 10⁹ to 3 × 10⁹ ml⁻¹) were washed twice in Tris buffer and finally suspended in 1 ml SMMD. Lysozyme (0.4 ml) was added. After 3 min incubation, 0.05 ml EDTA was blown in and incubation was continued for a further 18 min. Then a further 3 ml SMMD was added, the tube was centrifuged at 4000 g for 30 min and the deposit was suspended in 3 ml SMMD. The contents of the tube were then added to 100 ml medium G and incubated for 4 h, by which time the culture had visibly increased in density. Then 100 ml was centrifuged at 4000 g for 30 min and the deposit was suspended in 3 ml SMMD. At this stage the suspension contained about 2 × 10⁹ spheroplasts ml⁻¹. Two ml of the suspension was used as a control and the remaining 1 ml was mixed with 1 ml of the similarly treated fusion partner. Tubes were finally centrifuged at 4000 g for 30 min. All centrifugation was done at room temperature (about 25 °C).

Spheroplast lysate. This was made by suspending a pellet of spheroplasts in 3 ml deionized water. After visual clearing of the suspensions the osmolarity was adjusted to that of medium G with sucrose.

Enumeration of spheroplasts. This was done by counting in a haemocytometer under a phase-contrast microscope (Baltz, 1978; Okanishi *et al.*, 1974). Colony formation by osmotically stable cells, contained in a spheroplast suspension, was determined by diluting the suspension in deionized water and plating on MacConkey agar. The difference between the latter count and the viable count of the bacterial suspension immediately prior to spheroplast preparation was used as an independent check on haemocytometer counts. These counts also enabled an estimate of the efficiency of spheroplast conversion.

Electron microscopy of spheroplasts. Spheroplasts were fixed by a modification of the method of Sagara *et al.* (1971). This involved treatment with 3 % (v/v) glutaraldehyde in phosphate buffer (pH 7) containing 1 M-sucrose followed by post-fixation in 1 % (w/v) OsO₄ for 4 h; both steps were performed at 4 °C. The fixed material was dehydrated in a series of graded ethanol dilutions and embedded in Epon 812. Sections were cut on a LKB ultramicrotome using glass knives and viewed in a Philips EM300 electron microscope at an instrumental magnification of 42000 and an accelerating voltage of 60 kV.

Spheroplast fusion. The two methods of Fodor & Alföldi (1976) were used. (1) Calcium phosphate treatment. The spheroplast deposit after the final centrifugation was suspended in 2 ml SMMD. To this was added first 0.1 ml 0.02 M-KH₂PO₄ and then 0.1 ml 1.0 M-CaCl₂. After 1 min of gentle shaking the spheroplasts were plated. (2) PEG treatment. The final centrifuged pellet was suspended in 0.2 ml SMMD and

Table 1. *Strains of Providence alcalifaciens*

Strain	Relevant properties	Reference
P29 <i>nal-1</i>	P29 is the laboratory designation of NCTC 9211. Non-lysogenic despite many attempts to prove to the contrary. Susceptible to many Providence phages including the generalized transducing phage PL25. Spontaneous mutant resistant to at least 100 µg nalidixic acid ml ⁻¹	Coetzee (1963, 1975, and unpublished); Coetzee <i>et al.</i> (1966)
AK100	<i>trp-10</i> auxotroph of P29 <i>nal-1</i> obtained by NTG treatment	A. Kruger & J. N. Coetzee (unpublished)
AK115	<i>ilv-2</i> auxotroph of P29 <i>nal-1</i> obtained by NTG treatment	A. Kruger & J. N. Coetzee (unpublished)
AK200	<i>ade-1 his-22</i> auxotroph of P29 <i>nal-1</i> obtained by successive NTG treatments	A. Kruger & J. N. Coetzee (unpublished)
AK227	<i>ura-4 met-3</i> auxotroph of P29 <i>nal-1</i> obtained by successive NTG treatments	A. Kruger & J. N. Coetzee (unpublished)

NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

1.8 ml PEG was added. After gentle agitation for 1 min the spheroplasts were plated. All the above operations were done at room temperature (about 25 °C).

Plating of fusion mixtures and subsequent procedures. Suspensions were plated by transfer of 0.1 ml into 2.5 ml molten top-layer RRM agar maintained at 45 °C and poured over the surface of RRM plates. To determine regeneration frequencies of spheroplasts in fusion mixtures, further dilutions in SMMD were made and plated as above. Plates were incubated for 48 h. Bacterial colonies on the most heavily inoculated plates were only then subjected to selection by replication to MM or supplemented MM, while regenerated spheroplasts were counted on the more sparsely seeded RRM. Regeneration efficiency was expressed as the ratio of the number of colonies appearing on these plates to the number of spheroplasts plated.

Control experiments. To exclude possibilities of transformation or transfection, deoxyribonuclease I was added after spheroplast formation and was present in fluid media subsequently used as well as in RRM. Supernatants of samples of overnight broth cultures of strains as well as final spheroplasts suspensions, immediately prior to addition of fusion agents, were tested for lytic phage by plating on P29 *nal-1*. Experiments were also done with spheroplasts derived from each parent singly, with mixtures of parental bacteria (instead of their spheroplasts) and with mixtures of one of the parental bacteria, or spheroplasts derived from it, and a spheroplast lysate of the fusion partner. In other experiments, only the fusion agents were omitted from the experimental protocol.

Analysis of primary prototroph clones. Colonies arising on primary MM replica plates were picked off into individual tubes containing 10 ml nutrient broth and grown overnight. Dilutions were then made and plated on MacConkey agar to yield about 100 colonies per plate after incubation. These plates were then replicated to MM or supplemented MM. Samples of clones arising on the latter secondary replication media were again tested by the same procedure.

Segregation of unselected markers among recombinants. Colonies obtained on doubly supplemented MM primary replica plates were patched on the same medium. After overnight incubation, these plates were replicated to MM and MM supplemented with either or both of the two growth factors.

RESULTS

Properties of spheroplasts

Microscopic appearance. Examination in a phase-contrast microscope showed that more than 99% of organisms were converted to spherical forms as a result of the glycine and lysozyme-EDTA treatment. Electron microscope examination (Figs 1 to 4) revealed that the outer trilaminar component of the cell wall was broken in about 15% of spheroplasts. These breaks exposed areas of underlying cytoplasmic membrane. Many cell wall vesicles (Hofschneider & Martin, 1968) were also present.

Osmotic sensitivity. Suspension of a pellet of spheroplasts in deionized water caused rapid lysis of the spheroplasts accompanied by visual clearing of the suspension. This sensitivity, together with the special morphology and retention of some cell wall material, fits a definition of a spheroplast (McQuillen, 1960).

Figs 1 to 4. Thin sections of spheroplasts of *Providencia alcalifaciens* strain AK200 produced by combined glycine and lysozyme-EDTA treatment. Arrows indicate whorled cell wall material. The underlying cytoplasmic membrane is then exposed. Small vesicles, evident in all the photomicrographs, represent wall material in different section planes. Figure 1 shows a spheroplast devoid of cell wall, surrounded by others with possibly intact cell walls. Bar markers represent 100 nm.

Efficiency of spheroplast formation. This varied from 0.98 to 0.99 and the concentration of spheroplasts used in experiments was usually 2×10^9 ml⁻¹.

Spheroplast fusion

Microscopic appearance. Phase-contrast microscopy of spheroplast mixtures before centrifugation and addition of fusion agents revealed homogeneous suspensions of well-dispersed spheroplasts. Examination of final mixtures after centrifugation and treatment with either of the fusion agents showed many clumps consisting of 2 to 10 spheroplasts. Aggregation does not necessarily imply fusion (Schaeffer *et al.*, 1976) and an estimate of the number of genomes possibly contained in one fused cellular unit is not available (see Hopwood & Wright, 1978).

Regeneration efficiency of spheroplasts in fusion mixtures. This varied from 0.01 to 0.3 but in most experiments a value of about 0.1 was obtained. Due to aggregation produced by the fusion agents, these figures are lower than would be expected from untreated spheroplasts (see Baltz, 1978).

Genetic evidence of spheroplast fusion. Plaque-forming phage was not detected in supernatants of overnight broth cultures or in spheroplast preparations. Colonies which appeared on selection replica plates arose on the master RRM plates in the continued presence of deoxyribonuclease. Control experiments set up with the bacterial phase of single or double auxotroph pairs with or without spheroplast lysates of one of the partners, often had 1 to 10 clones on MM or singly supplemented MM replica plates, respectively (not shown). This corresponded to maximum reversion frequencies of about 5×10^{-8} per locus. In contrast, control experiments with single spheroplast suspensions never had growth on replica plates. This may be due to the high efficiency of spheroplast formation coupled to a low frequency of spheroplast regeneration (see above). Clones arose on test selective replica plates only when use of a fusion agent was included (not shown) and this implicated the action of fusion agents in the results obtained. No consistent difference was detected between the results obtained using either PEG or calcium phosphate as the fusion agent. With *Bacillus megaterium*, PEG treatment of protoplasts sometimes produced higher yields of protoplasts than did the latter agent (Fodor & Alföldi, 1976). For the present purposes, the two agents will be regarded as having identical effects.

The number of colonies which arose on primary replica plates of tests varied according to the selection applied (Table 2). Recombination frequencies of 3.8×10^{-6} to 1.7×10^{-7} per spheroplast in mixtures prior to addition of fusion agent are lower than those recorded for protoplasts of *Bacillus subtilis* (Schaeffer *et al.*, 1976) and compare even less favourably with those of *Streptomyces* spp. (Hopwood *et al.*, 1977; Hopwood & Wright, 1978; Baltz, 1978). Assuming a spheroplast regeneration frequency of 0.1 (see above), this implies that, at most, 0.003% of spheroplasts which regenerated a cell wall were recombinants. Excellent reproducibility of results was demonstrated in numerous experiments. The crowding effect noted by Schaeffer *et al.* (1976) and Hopwood *et al.* (1977) was not encountered. Invariably the number of recombinants which arose on a replica plate bore a direct relation to the concentration of the fusion mixture plated. Obviously we were not dealing with the total number of heteroploids produced and this suggested that the growth was the result of post-fusional genetic recombination rather than repeated division of initial heteroploid cells, as mutual complementation would have rendered the latter independent of growth supplements.

Analysis of primary colonies. Fifty colonies, each arising on the primary MM replica plates of fusion crosses AK200 $\times$ AK227 or AK200 $\times$ AK100 (Table 2) were examined. Two types of clones were encountered (not shown). The first (and most frequent) consisted of mixed populations of prototrophs and one, or very rarely, two other recombinants. The second type of clone only yielded prototrophs. Parental types were never isolated. These types of prototroph clones were found in both the above fusion experiments in about equal

Table 2. *Analysis of recombinant colonies which arose on primary selective replica plates in spheroplast fusion experiments*

Fusion experiments were done as described in Methods. The concentration of mixed spheroplasts, before fusion, was 2×10^8 ml⁻¹. Parental spheroplasts, singly, produced no colonies on replica plates of control experiments. For results of other control experiments, see text.

Cross	Supplementation of MM replica plates	No. of clones	10 ⁶ × Recombination frequency*	Minimum no. of crossovers required
AK200 × AK227	None	34	0.17	2 or 4†
	Adenine	338	1.7	2 or 4†
	Histidine	765	3.8	2‡
	Uracil	727	3.6	2 or 4†
	Methionine	359	1.8	2 or 4†
	Adenine, methionine	710	3.5	2†
	Uracil, histidine	625	3.1	2‡
AK100 × AK200	None	303	1.5	2 or 4‡
	Adenine	701	3.5	2‡
	Histidine	748	3.7	2‡
AK100 × AK115	None	690	3.5	2

*Per spheroplast in mixture immediately prior to fusion.

† Based on locus sequences *ade his ura met*, *ade ura his met* or *ade ura met his*.

‡ Based on locus sequences *ade trp his* or *trp ade his*.

Table 3. *Segregation of unselected markers among recombinants from a AK200 × AK227 fusion experiment*

Colonies obtained on MM supplemented with two of the four growth factors, were patched on the same medium. After overnight incubation, these plates were replicated on MM and on MM containing either growth factor or both.

Unselected markers	Percentage of colonies having indicated constitution			
	AK200 parental type	AK227 parental type	Recombinant	Recombinant
	(-/+)	(+/-)	(+/+)	(-/-)
<i>ade/met</i>	15	25	25	35
<i>his/ura</i>	42	35	8	15

numbers. A limited amount of testing (see Methods) of the secondary colonies arising from the above primary clones revealed them to be stable (see Schaeffer *et al.*, 1976).

Segregation of unselected markers. The requirements of partial prototrophs for the two growth factors which were present during selection (Table 2, lines 6, 7) were determined by replica-plating (Table 3). The two parental and two recombinant types were encountered in both experiments. The results of these experiments independently support those above which suggest that the fusion-induced recombinants are haploid.

DISCUSSION

Control experiments ruled out possibilities of auxotroph reversion, conjugation, trans-formation, transfection or transduction as explanations of the results. The fact that colonies arose on test plates only after treatment with protoplast fusion agents implicated spheroplast fusion as the cause of the results obtained. The effect of centrifugation in promoting protoplast adhesion (Ferenczy *et al.*, 1976; Fodor & Alföldi, 1976) was not investigated independently although centrifugation of mixed spheroplasts formed part of the experimental protocol.

The strategy employed in making spheroplasts was to attack the peptidoglycan layer of the cell wall at two different sites. Glycine apparently acts by interfering with cross-linking

of the muramic acid peptide molecules (Strominger, 1968), while lysozyme destroys certain glycoside linkages between the amino sugars (Rogers & Perkins, 1968) once the former have been exposed, in Gram-negative bacteria, by the chelating action of EDTA (Repaske, 1956, 1958). Unlike lysozyme-EDTA treatment or glycine treatment, alone, which convert Providence cells to spheroplasts possessing an entire outer integument (Van Rensburg, 1969), their combined action often resulted in rupture of this structure with exposure of the underlying cytoplasmic membrane (Figs 1 to 4). The combined action of glycine and lysozyme without EDTA has been used by Sagara *et al.* (1971) and Baltz (1978) for the formation of protoplasts in Gram-positive *Streptomyces* spp. Birdsell & Cota-Robles (1967) and Costerton *et al.* (1967) produced spheroplasts of *Escherichia coli* and a marine pseudomonad, respectively, by using only lysozyme-EDTA treatment. These spheroplasts had large breaks in the outer cell wall integument.

If the fusion of spheroplasts demonstrated here took place in the exposed areas of cytoplasmic membrane, then the limited areas available would result in frequencies of spheroplast recombination lower than those encountered with protoplast fusion. Pontecorvo *et al.* (1977) have suggested that in human fibroblasts, PEG-mediated fusion occurs in very restricted areas at or near the tips of the filamentous processes of these cells.

Quite apart from the particular method used for spheroplast preparation or the fusion techniques employed, recombinant recovery is also (and finally) dependent on the regeneration of spheroplasts to cells (Baltz, 1978). As selection was only applied after regeneration, the constraint of only complementing (fused) spheroplasts being able to regenerate cell wall was removed (see Fodor & Alföldi, 1976). The estimated frequency of spheroplast regeneration after fusion treatment (about 0.1) was of the same order as recorded for *Bacillus* spp. protoplasts (Fodor & Alföldi, 1976; Schaeffer *et al.*, 1976) but decidedly lower than that recorded for *Streptomyces* spp. by Okanishi *et al.* (1974), Hopwood *et al.* (1977) and Baltz (1978). Toxicity of one of the treatments could be involved (see Baltz, 1978) but frequencies presented here are lower than would be expected as spheroplasts and not protoplasts were used. The former possess remnants of cell wall which could act as primers for new cell wall elements during the regeneration process (Hofschneider & Martin, 1968; Landman, 1968).

Analysis of primary prototrophic clones suggested that, in all cases, the initial heteroploid phase was soon succeeded by one or more haploid recombinants. Fodor & Alföldi (1976) found a class of primary prototrophic clones of *Bacillus subtilis* which gave rise to mixed populations in which the prototrophic elements were unstable, but we found no such class.

Prototroph recombinants were more frequent in double $\times$ single auxotrophic spheroplast fusions, and in single $\times$ single auxotroph spheroplast matings, than in fusions where both partners were double auxotrophs (Table 2). This was expected since in the first two fusion types mentioned two crossover events (depending on the relationship of the chromosomal loci to one another) would restore one of the parental genomes to prototrophy, while in the latter case four crossovers would be required. The locus order *ade-1 ura-4 his-22 met-3* best fits the results of the first cross listed in Table 2. Similarly the order derived from the second cross could be *ade-1 trp-10 his-22*. Although the particular genes corresponding to the above loci have not been identified, the locus order corresponds to that of the *Proteus mirabilis* chromosome (Coetzee, 1979*a, b*). Providence and *Proteus* strains are closely related (Coetzee, 1972). If this comparison is valid, some of the recombinants isolated here involved regions separated by about half the length of the chromosome. This indicates extensive initial diploidy. Fusion crosses may be useful in generating many combinations of parental genes (see Hopwood & Wright, 1978).

Although conventional genetic analysis of *Providence alcalifaciens* is proceeding by means of R plasmids capable of chromosome mobilization (A. Kruger & J. N. Coetzee, unpublished), the work presented here resembles that of Fodor & Alföldi (1976) for *Bacillus megaterium* in that it precedes reports of chromosomal recombination brought about by conjugation.

The prime advantage of genetic recombination through spheroplast fusion is that it opens the way for genetic analysis in organisms lacking sex factors. Another advantage is that counterselection for one of the parents is not required. A third benefit is the general loosening of linkage (Hopwood *et al.*, 1977) resulting from the temporary (at least) confrontation and complementation, in the same cell, of possibly entire parental genomes. The use of this technique to promote interspecies recombination is being studied.

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