Computer-assisted chromosome mapping in Staphylococcus aureus by protoplast fusion

Mark Lloyd Stahl
Iowa State University

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COMPUTER-ASSISTED CHROMOSOME MAPPING IN STAPHYLOCOCCUS AUREUS BY PROTOPLAST FUSION

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Computer-assisted chromosome mapping in

Staphylococcus aureus by

protoplast fusion

by

Mark Lloyd Stahl

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INTRODUCTION

Despite the availability of generalized transduction (Morse, 1959; Ritz and Baldwin, 1962) and transformation (Lindberg et al., 1972; Pattee and Neveln, 1975) as methods of genetic analysis, knowledge of the genomic organization in Staphylococcus aureus NCTC 8325 has been limited. Transformation analyses resulted in the construction of 3 distinct linkage groups; however, it has not been possible to define the relationship of these linkage groups to one another on the S. aureus chromosome. Because of the size and complexity of the established linkage groups, mapping new markers by transformation analysis can be a laborious process. Consequently, there has been interest in developing other methods of genetic exchange that might prove useful for chromosome mapping in S. aureus.

Engel et al. (1980) have reported the conjugal transfer of plasmid pRl405 from streptococci to S. aureus and between strains of S. aureus. No evidence, thus far, of chromosome mobilization by this plasmid has been obtained, however. Genetic recombination by protoplast fusion has been described in several procaryotic species including Bacillus subtilis (Schaeffer et al., 1976), Bacillus megaterium (Fodor and Alfoldi, 1976), Brevibacterium flavum (Keneko and Sakaguchi, 1979), Escherichia coli (Tsenin et al., 1979), Providence
*alcalifaciens* (Coetzee et al., 1979), *Staphylococcus aureus* (Hirachi et al., 1979, 1980; Gotz et al., 1981), several *Streptomyces* spp. (Hopwood et al., 1977; Baltz, 1978; Godfrey et al., 1978), and lactic streptococci (Gasson, 1980). In addition to chromosomal recombination, Gotz et al. (1981) have demonstrated plasmid transfer among staphylococci.

Protoplast fusion is unique as a mode of genetic exchange in procaryotes because the transfer of genetic information is bidirectional and intact chromosomes are combined in the same cytoplasm at high frequencies. In addition, the survival of each viable parent in the cross must be selected against for the analysis of the desired classes of recombinant phenotypes.

The interest in protoplast fusion with *S. aureus* centered primarily on its potential as a supplementary technique in chromosome mapping. The specific goals of this research project included: 1) developing a protoplast fusion procedure with *S. aureus* for the analysis of selected recombinant phenotypes, 2) using the analysis procedure to predict the location of unmapped chromosomal markers, which then could be mapped quantitatively by transformation, and 3) obtaining evidence by protoplast fusion for the orientation of the 3 linkage groups on a circular map.
LITERATURE REVIEW

Chromosome Mapping in Staphylococcus aureus

Generalized transduction in Staphylococcus aureus (Morse, 1959) has been used extensively for fine-structure genetic mapping within specific regions of the chromosome (Ritz and Baldwin, 1962; Kloos and Pattee, 1965; Smith and Pattee, 1967; Proctor and Kloos, 1970; Pattee et al., 1974). However, because of the limiting headful size (30 megadaltons) of staphylococcal generalized transducing phage (Lacey, 1975) and apparent homogeneity of transducing fragments (Pattee et al., 1968), generalized transduction is only of limited value when studying gross chromosomal organization.

Lindberg et al. (1972) found that physiologically competent cultures of S. aureus NCTC 8325, a phage group III strain, take up genetically active DNA when exposed to a combination of heat shock and 0.1 M CaCl$_2$. In early studies (Sjostrom et al., 1973; Sjostrom and Philipson, 1974), the competent transforming state of the cell was attributed to the expression of a bacteriophage early gene; the recipient culture had to be lysogenic for either $\phi 11$ or 83A for the uptake of genetically active DNA. More recent studies (Thompson and Pattee, 1977) have shown that most coagulase positive staphylococci can be transformed and all serological
group B bacteriophage can confer competence activity. It now appears almost certain that the phage attachment organelle or a part of it must be bound to the recipient cell for DNA uptake to occur (Thompson and Pattee, 1977; Thompson and Pattee, 1981; Birmingham and Pattee, 1981).

Pattee and Neveln (1975) constructed 3 linkage groups of chromosomal loci by transformation analysis; markers within each of the linkage groups were cotransformable while markers from different linkage groups were not. A considerable amount of evidence also was presented against congression (false linkage due to cotransformation of markers on separate fragments of DNA) being responsible for contributing to the transformation-derived linkages.

Since 1975, the size and detail of these linkage groups has progressed further. In particular, the development of Tn551 insertional mutagenesis in *S. aureus* contributed significantly to the extension of the 3 linkage groups (Pattee, 1981). Tn551 is a transposable element which carries an *ermB* determinant that confers constitutive erythromycin resistance and is capable of insertion into many different chromosomal sites (Novick, 1967; Novick, 1974; Pattee et al., 1977; Novick et al., 1979; Pattee, 1981). Although some Tn551 insertions imposed an auxotrophic phenotype on the organism (as a consequence of insertional mutation), all Tn551 inserts
resulted in the Em\(^{r}\) phenotype. This last fact placed certain limitations on the kinds of genetic crosses that could be performed. Because of these constraints, and the added complexity and increased size of the 3 linkage groups, mapping new markers by transformation has become more laborious.

Pattee and Glatz (1980) divided the linkage groups into overlapping segments (Figure 1). Each segment was bound by a pair of readily scored or selected markers that exhibited reproducible but usually low cotransformation frequencies. Each segment was screened for a chromosomal entA\(^{+}\) marker, which eventually was mapped near bla\(^{+}\) in linkage group III. This process was necessary for mapping markers which were difficult to select; and it was successful only if the marker mapped within or near the established linkage groups. In fact, several markers which have been screened by transformation with similar approaches, have not shown linkage to markers within the known linkage groups and have been designated as orphans (personal communication, P. A. Pattee, Iowa State University, Ames, Iowa).

Protoplast Fusion

Chromosomal recombination by polyethylene glycol (PEG)-induced protoplast fusion was first described for procaryotes by Schaeffer et al. (1976) with *Bacillus subtilis* and
Figure 1. The three known linkage groups of the S. aureus chromosome (Pattee and Neveln, 1975; Pattee, 1976; Kuhl et al., 1978; Pattee, 1980; Pattee and Glatz, 1980). The markers used in this study and the phenotypes they confer are listed in Table 3.
Fodor and Alfoldi (1976) with Bacillus megaterium. In con­
currence with more detailed genetic studies, conditions for
fusion and recombination have since been optimized for both
species (Levi et al., 1977; Fodor et al., 1978; Fodor and
Alfoldi, 1979; Gabor and Hotchkiss, 1979; Hotchkiss and Gabor,
1980).

Detailed genetic studies by protoplast fusion also were
reported with several Streptomyces spp. (Hopwood et al., 1977;
Baltz, 1978; Godfrey et al., 1978; Hopwood and Wright, 1978,
1979). Probably the most significant advances in under­
standing genetic exchange by protoplast fusion were made with
Streptomyces coelicolor by Hopwood and coworkers (Hopwood

Chromosomal recombination by protoplast fusion has also
been demonstrated in other gram positive and gram negative
species including Brevibacterium flavum (Keneko and Sakaguchi,
1979), Escherichia coli (Tsenin et al., 1979), Providence
alcalifaciens (Coetzee et al., 1979), Staphylococcus aureus
(Hirachi et al., 1979, 1980; Gotz et al., 1981), and lactic
streptococci (Gasson, 1980). To date, however, more detailed
 genetic studies by fusion have not been pursued with these
systems.

Recently, Hopwood (1981) published a comprehensive review
of the genetic applications and uses of protoplasts that
included a summary of the conditions necessary to demonstrate protoplast fusion and chromosomal recombination among procaryotes. This review will provide a brief summary of the experimental conditions necessary for fusion and recombination, but will emphasize that part of the literature associated with genetic interactions and analysis by fusion.

**Preparation of protoplasts**

Protoplasts usually are made from bacteria by exposing cells, grown under the appropriate conditions, to an enzyme which is capable of degrading the peptidoglycan layers of the cell wall. When bacteria are grown in hypotonic media, a high intracellular osmotic pressure is inherent. To protect the integrity of the protoplasts during cell wall removal, they must be suspended in a hypertonic buffer solution. Many investigators have used modifications of the sucrose-magnesium-maleate buffer of Wyrick and Rogers (1973) which contains 0.3 M sucrose as the osmotic stabilizing ingredient.

Protoplasts were made from *B. subtilis* (Schaeffer et al., 1976) and *B. megaterium* (Fodor and Alfoldi, 1976) by treating washed cells with lysozyme, while protoplasts from other gram positive bacteria were more difficult to obtain. Usually, a treatment during the growth of the culture to be protoplasted was needed to destabilize the crosslinking of the peptidoglycan layers within the cell walls. High
concentrations of glycine (0.8-3.5%) added to the culture medium have been reported to increase the sensitivity of Streptomyces spp. to lysozyme (Sagara et al., 1971; Okanishi et al., 1974; Hopwood et al., 1977). The optimum concentration used was found to be species-dependent. In Brevibacterium flavum (Keneko and Sakaguchi, 1979), sublethal concentrations of penicillin were added to logarithmically growing cells to make them more susceptible to lysozyme treatment. Protoplasts were made from S. aureus by treating the cells with lysostaphin (Hirachi et al., 1979, 1980; Gotz et al., 1981). No pre-enzyme treatment of the growing cells was necessary to obtain good yields of protoplasts.

The production of true protoplasts from gram negative bacteria has met with limited success. Dissolution of the peptidoglycan layers must be accompanied by removal of the outer membrane. In E. coli (Tsenin et al., 1979) and P. alcalifaciens (Coetzee et al., 1979) protoplasts were made by adding glycine to the culture medium and treating the washed cells with a combination of EDTA and lysozyme.

**Polyethylene glycol-induced protoplast fusion**

PEG was first used as a fusogen for plant protoplasts (Kao and Michaylak, 1974; Kao et al., 1974) and the following observations were made for its use: 1) a wide range of PEG polymer molecular weights were effective (from about 1000 up
to at least 6000); 2) concentrated solutions (50% w/v) were much more effective than dilute solutions (25% w/v); 3) solutions of PEG of different molecular weight were equally effective at the same concentration; 4) the appropriate concentration of divalent calcium ions was necessary for fusion to occur; and 5) protoplast adhesions occurred almost instantaneously upon the addition of PEG solution.

Pontecorvo (1975) was the first to use PEG to induce fusion in mammalian cells by treating human fibroblast cells with 50% (w/v) PEG 6000. It was later shown that a solution containing both 41-47% (w/v) PEG 1000 and 15% (v/v) dimethyl sulfoxide (a fusion enhancer) stimulated higher fusion frequencies (Norwood et al., 1976).

Protoplast fusion and chromosomal recombination were induced in B. subtilis (Schaeffer et al., 1976) and B. megaterium (Fodor and Alfoldi, 1976) by using PEG 6000 at a final concentration of 36% (w/v). In a later study, Gabor and Hotchkiss (1979) found that changes in PEG molecular weight had little effect on the frequency of recombination in B. subtilis fusions. In these experiments, the frequency of recombination was defined as the number of recombinants per protoplast plated onto the regeneration medium.

Optimum frequencies of recombination were observed when S. coelicolor protoplasts were fused in a solution containing a final concentration of 42% (w/v) PEG 1540 plus 15% (w/v)
DMSO (Hopwood et al., 1977). Later studies proved that equally high recombination frequencies were observed using either 42% (w/v) PEG 1000 plus 15% (v/v) DMSO or 50% (v/v) PEG 1000 (Hopwood and Wright, 1979). The fusion enhancing effect of DMSO could be duplicated by increasing an apparently suboptimal PEG concentration.

Hirachi et al. (1979, 1980) found that 50% (w/v) PEG 6000 was more effective than PEG 4000 or PEG 1000 at the same concentration in fusion studies with S. aureus L-forms. Gotz et al. (1981) demonstrated fusion and chromosome recombination with S. aureus protoplasts only when a combination of calcium chloride (0.16 M) and PEG 6000 (approximately 30% w/v) was used. If PEG was used alone, only plasmid transfer between protoplasts could be demonstrated.

Senda et al. (1980) found that the fusion of plant protoplasts in the presence of PEG is greatly enhanced by a rise in temperature. It was speculated that membrane fluidity was important in regulating the fusion process. In procaryotes, variations in the time and temperature at which fusion mixtures were held seemed to have minimal effects on observed recombination frequencies. Bacillus subtilus fusions were incubated at 0°C or at room temperature (Schaeffer et al., 1976; Gabor and Hotchkiss, 1979) and Streptomyces spp. fusions were incubated at room temperature.
(Hopwood et al., 1977; Baltz, 1978; Godfrey et al., 1978). In these same fusion systems, the time at which fusion mixtures were held proved to be relatively unimportant and varied between 1 and 3 min.

**Regeneration of protoplasts**

Protoplast regeneration in bacteria to the cellular state requires a set of nutritional and physical conditions which may be unique to a single species (Hopwood, 1981).

Schaeffer et al. (1976) used a medium developed by Wyrick and Rogers (1973) to regenerate fused protoplasts of *B. subtilis*. The medium contained the following ingredients per liter of water: agar, 8 g; acid-hydrolyzed casein, 5 g; K$_2$HPO$_4$, 3.5 g; KH$_2$PO$_4$, 1.5 g; 81 g sodium succinate (pH 7.3); 1.9 g MgCl$_2$; glucose, 5 g; tryptophan, 0.1 g; and 5 ml of heat inactivated (56°C for 30 min) filter sterilized horse serum. Gabor and Hotchkiss (1979) modified the medium to include 53.5 g sodium succinate, 20 g agar, 5 g gelatin, and calf serum was substituted for horse serum. The use of this regeneration medium in conjunction with 1% serum albumin in the suspending fluids resulted in almost 50% regeneration frequencies of fused *B. subtilis* protoplasts. Regeneration frequencies were estimated by comparing colony counts on the regeneration medium to prefusion hemocytometer counts of
the protoplasts. On media of low agar concentration (0.8 to 1.5%), early-forming colonies inhibited or "crowded out" the regeneration of protoplasts nearby (Landman and DeCastro-Costa, 1976; DeCastro-Costa and Landman, 1977; Hopwood et al., 1977). Landman and DeCastro-Costa (1976) and DeCastro-Costa and Landman (1977) alleviated this crowding effect for *B. subtilis* protoplasts by increasing the agar concentration in the regeneration medium to 2-3%.

As contrasted with the surface spreading of fused *B. subtilis* protoplasts on a regeneration medium, Fodor and Alfoldi (1976) regenerated fused protoplasts of *B. megaterium* in soft agar overlays. Sucrose replaced sodium succinate as the osmotic stabilizing agent.

The development of protoplast fusion systems in *Streptomyces* spp. (Hopwood et al., 1977; Baltz, 1978; Godfrey et al., 1978) was based on the techniques of protoplast regeneration developed by Okanishi et al. (1974). Protoplasts were regenerated on the surface of synthetic media supplemented with 0.01% casamino acids. The appropriate ratio and concentrations of magnesium and calcium ions in the medium also were critical for high regeneration frequencies.
Genetic analysis by protoplast fusion

Hopwood (1981) has hypothesized that shortly after PEG-induced protoplast fusion a diploidal or a polyploidal fusion body is formed. From these fusion bodies, random recombination and further segregation of chromosomal material occurs until haploid recombinants and parental phenotypes are formed. If the previous assumptions are true, then all of the markers from each parent genome should have an equal chance for inheritance, regardless of their chromosomal loci. Other established modes of genetic exchange, including generalized transduction, transformation, and conjugation, are mediated strictly by a unidirectional transfer of only fragments of the donor chromosome.

In Streptomyces coelicolor, Hopwood and Wright (1978) found that fusions from mixtures of 4 parental genotypes resulted in the production of recombinants inheriting alleles from 3 or 4 genomes. Hopwood (1981) has also seen differences in the heterozygotes produced by conjugal matings and protoplast fusions between an NF donor strain and an SPCI recipient strain of S. coelicolor. The NF donor strain carries the SCPl sex plasmid integrated into the chromosome and through conjugal mating with an SPCI recipient strain, it is capable of mobilizing markers on the chromosome. When mated, markers closely linked to the SPCI integration site in
the donor strain were transferred to the recipient strain at high frequencies. Markers that were distantly linked to the integration site were transferred at much lower frequencies. When these same 2 strains were fused, all alleles from both parents were inherited at equal frequencies among the recombinants. No selection was made during regeneration or during the analysis of recombinant phenotypes.

When compared to a well-developed chromosomal map, 25% of the recombinants from a six-factor fusion of *S. coelicolor* belonged to multiple crossover classes (Hopwood and Wright, 1978). Regeneration was nonselective and the recombinants were sorted from parental genotypes by a nonselective analysis of spores harvested from regeneration plates. Approximately 11% of the total spores harvested were recombinants and 57 of the possible 62 recombinant phenotypes occurred. When the same 2 strains were crossed by a conjugal mating only 2% of the recombinants belonged to multiple crossover classes. Although crossovers per unit map length were more frequent by protoplast fusion, the fusion data were consistent with known linkage relationships.

Fusion-derived recombinants of *B. megaterium* (Fodor and Alfoldi, 1979) were obtained by directly selecting against at least one marker from each parent during regeneration. Since no genetic map is known, the authors hoped to use protoplast
fusion for chromosomal mapping. However, no significant conclusions could be made from the data that were presented. Depending upon the nutritional substitutions made during the growth of the parent strains before protoplast formation, totally different classes of fusion-derived recombinants were obtained.

In protoplast fusions with B. subtilis (Schaeffer et al., 1976), the growth on nonselective regeneration plates were replicated onto selective media. Clones were picked from these plates and transferred onto media of the same composition in an ordered array on master plates. Unselected markers involved in the cross were scored by replicating the master plates onto minimal media of the appropriate composition. After the results from 5 different selections of a six-factor cross were examined, predicted linkages (based on fewer crossovers between closely linked markers as compared to more distant markers) coincided with the arrangement of the markers on the known linkage map.

In later work (Hotchkiss and Gabor, 1980), the authors focused their attention on biparental or diploid regenerants derived from B. subtilis fusions. Well-isolated colonies from plates of nonselective regeneration medium were picked, and of these, biparentals were identified by the ability to grow only on media selective for the growth of each parent.
Normally, biparentals comprised from 1 to 4% of the total number of regenerated colonies. Complementation between auxotrophic and prototrophic alleles did not occur as only one parent chromosome was expressed. When any one biparental isolate was subcultured in nonselective liquid medium, biparentals could be isolated which expressed the alleles of the formerly unexpressed chromosome. Genetically haploid recombinants and parental phenotypes also were isolated from these same subcultures. Further evidence for the existence of a diploid state was presented as markers from the unexpressed chromosome of a biparental were transformed into and were expressed in a genetically haploid background.

**Fusions between nonviable protoplasts**

One of the most useful applications of protoplast fusion is the ability to combine markers that cannot be directly selected in the same genetic background (Hopwood, 1981). This is made possible because of the high frequencies of recombination observed with most fusion systems (up to 1-10% of the total viable regenerants). Presumably, even higher percentages of recombinants could be generated if one or both of the parental types could be eliminated during regeneration.

In *B. subtilis*, a streptomycin-sensitive parent was killed with streptomycin and then was fused with a resistant strain.
(Levi et al., 1977), but the number of recombinants recovered suffered a 10-fold decrease when compared to control fusions.

In B. megaterium, heat-killed protoplasts of one parent were fused with a viable parent (Fodor et al., 1978). However, when compared with control fusions between viable parents, the recombination frequencies were generally lower.

An effective method for recombinant enrichment in S. coelicolor protoplast fusions has been to irradiate one or both parents with ultraviolet (UV) radiation (Hopwood and Wright, 1979). Protoplasts with lethal doses of UV radiation only survive if they fuse and recombine with other killed protoplasts. UV radiation also increased the proportion of recombinants with multiple crossovers. The majority of recombinants inherited individual "donor" markers and showed a complete loss of linkage with other markers known to be closely linked (Hopwood and Wright, 1981).
MATERIALS AND METHODS

Bacteria

The strains of Staphylococcus aureus used in this study are listed in Table 1, along with their genotypes and origins. Some of the strains carry chromosomal insertions of Tn551, a transposable element that carries an \textit{ermB}^+ determinant which confers constitutive erythromycin resistance (Novick, 1967; Novick, 1974; Pattee et al., 1977; Novick et al., 1979; Pattee, 1981). The Tn551 insertion mutations used in this study generally fall into 2 categories: 1) "silent" insertions, where the nature of the chromosomal locus into which the element is inserted, if any, is unknown; each is designated by the Greek letter omega followed by a number (e.g. Ω34[Chr::Tn551]) and 2) other insertions (e.g. purC193::Tn551), in which the element has inserted into a known determinant which, in this particular example, is responsible for a biosynthetic capability. Pattee (1981) provides a detailed description of Tn551 insertion mutagenesis. All cultures were maintained on brain heart infusion (BHI, Difco) agar slants and stored at 4 C. A second set of stock cultures were maintained at -70 C in GL broth plus 10% glycerol. GL broth consisted of 3 g casamino acids (Difco), 3 g yeast extract (Difco), 5.9 g NaCl, 3.3 ml of 60% sodium lactate, and 4 ml of 25% glycerol, all per liter of deionized water (Novick et al., 1979).
Table 1. Designation, genotype, and origin of strains of *Staphylococcus aureus*

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<td>Pattee and Neveln, 1975</td>
</tr>
<tr>
<td>ISP2</td>
<td>8325nov-142 pig-131</td>
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</tr>
<tr>
<td>ISP5</td>
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<td>ISP32</td>
<td>8325lys-115 pig-131</td>
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<td>ISP37</td>
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<td>Strain 655 DNA x ISP5&lt;sup&gt;a&lt;/sup&gt;; Proctor and Kloos, 1970</td>
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<td>Pattee and Glatz, 1980</td>
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<td>Pattee et al., 1977</td>
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<td>Pattee and Glatz, 1980</td>
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<td>phage 80A/ISP161 x ISP87&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>ISP300</td>
<td>8325(80a)&lt;sup&gt;Ω34[Chr::Tn551]&lt;/sup&gt; pig-131</td>
<td>RN2573&lt;sup&gt;c&lt;/sup&gt;; Pattee and Glatz, 1980</td>
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<td>ISP364</td>
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<td>RN1855; Pattee and Glatz, 1980</td>
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</table>

<sup>a</sup> ISP5 was transformed with strain 655 DNA.

<sup>b</sup> ISP87 was transduced with a phage 80A lysate prepared on ISP161. The methods have been described (Kloos and Pattee, 1965).

<sup>c</sup> Richard P. Novick stock culture collection, Department of Plasmid Biology, The Public Health Research Institute of the City of New York, Inc., New York, NY.
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<tr>
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<td>Iordancescu and Surdeanu, 1976</td>
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<td>ISP483</td>
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</tr>
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\(^d\) Isolated by Tn551 mutagenesis by the method of Pattee (1981).

\(^e\) An ISP483 x ISP661 protoplast fusion cross. The methods for simulating a unidirectional transfer of genetic information were not used.
Table 1 (Continued)

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<td>ISP803</td>
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fErythromycin-sensitive derivative of Tn551 obtained by penicillin-counterselection with ISP673 (unpublished method).

gAn ISP849 x ISP459 protoplast fusion cross. The methods for simulating a unidirectional transfer of genetic information were used as ISP849 was the irradiated donor parent.
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Media and Reagents

All dehydrated commercial media were supplemented with thymine (20 μg/ml), adenine, guanine, cytosine, and uracil (5 μg each/ml) Pattee and Neveln, 1975). The composition of complete defined synthetic agar (CDS, Table 2) was modified by omitting the appropriate amino acids, purines, pyrimidines and by adding antibiotics as needed (Brown and Pattee, 1980; Pattee and Glatz, 1980). Antibiotic resistance phenotypes were selected by adding the appropriate concentration of antibiotic to BHI agar. The selectable genetic markers studied, their phenotypes, and the composition of the media used to select and score them, are listed in Table 3.

Protoplasts were formed in sucrose-magnesium-tris buffer (100mM tris(hydroxymethyl)amino methane, 40mM MgSO₄, 0.8M sucrose, pH 7.6, SMTB). Deoxyribonuclease (DNase I, Sigma) stock solution (3 mg/ml) was prepared according to the manufacturer's instructions. Lysostaphin (Sigma) was dissolved at a concentration of 1 mg/ml in a buffer containing 600 mg tris(hydroxymethyl)amino methane, 870 mg NaCl, and 100 ml deionized water at pH 7.5. Both the DNase and lysostaphin stocks were filter sterilized, dispensed into 1-ml aliquots, and stored at -20°C. Protoplasts were fused in 60% (v/v) polyethylene glycol (PEG; molecular weight 400; Sigma) in SMTB. Regeneration (R) medium consisted of tryptase soy broth (TSB; BBL), 30 g; sucrose (Sigma), 273 g;
Table 2. Composition of complete defined synthetic agar

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<thead>
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<th>Ingredient</th>
<th>Amount b</th>
<th>Ingredient</th>
<th>Amount b</th>
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<td>Glucose</td>
<td>5 gm</td>
<td>amino acids:</td>
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<td>Salts:</td>
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<td>L-glutamic acid</td>
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<td>K2HPO4</td>
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<td>L-serine</td>
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<td>K2HPO4</td>
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<td>L-methionine</td>
<td>10 mg</td>
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<td>MgSO4</td>
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<td>L-alanine</td>
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<td>(NH4)2SO4</td>
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<td>L-threonine</td>
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<td>glycine</td>
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<tr>
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<td>L-cystine</td>
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<tr>
<td>Thymine</td>
<td>20 mg</td>
<td>Difco agar</td>
<td>15 gm</td>
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</table>

Deionized water 1000 ml

a Synthetic media were prepared by combining required volumes of each stock; these mixtures were filter-sterilized and combined with the Difco agar which had been previously autoclaved (121 C, 15 min) with the remaining water and cooled to 50 C.

b Final concentration per liter.
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<th>Medium composition</th>
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<td>-threonine in CDS agar</td>
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<td>trpE85</td>
<td>Trp&lt;sup&gt;−&lt;/sup&gt; tryptophan requirement</td>
<td>-tryptophan in CDS agar</td>
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<td>uraA141</td>
<td>Ura&lt;sup&gt;−&lt;/sup&gt; uracil requirement</td>
<td>-pyrimidines in CDS agar</td>
</tr>
<tr>
<td>tyrB282</td>
<td>Tyr&lt;sup&gt;−&lt;/sup&gt; tyrosine requirement</td>
<td>-tyrosine in CDS agar</td>
</tr>
<tr>
<td>ermB321</td>
<td>Em&lt;sup&gt;s&lt;/sup&gt; erythromycin sensitive mutation of ermB&lt;sup&gt;+&lt;/sup&gt; in Tn551</td>
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<tr>
<td>nov-142</td>
<td>Nov&lt;sup&gt;r&lt;/sup&gt; novobiocin resistance</td>
<td>+10 ug novobiocin per ml in BHI agar</td>
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<tr>
<td>mec-4916</td>
<td>Mec&lt;sup&gt;r&lt;/sup&gt; methicillin resistance</td>
<td>+6.25 ug methicillin per ml +5% NaCl (Kuhl et al., 1978) in trypticase soy agar (BBL)</td>
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<td>ilv-129</td>
<td>Ilv&lt;sup&gt;−&lt;/sup&gt; isoleucine requirement</td>
<td>-isoleucine and leucine; valine reduced to 20 ug/ml; +1% sodium pyruvate (Brown and Pattee, 1980)</td>
</tr>
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<td>tet-3490</td>
<td>Tet&lt;sup&gt;r&lt;/sup&gt; tetracycline resistance</td>
<td>+1 ug (10 ug)&lt;sup&gt;a&lt;/sup&gt; of tetracycline per ml in BHI agar</td>
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<tr>
<td>Th551</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt; erythromycin resistance</td>
<td>+1 ug (10 ug)&lt;sup&gt;a&lt;/sup&gt; of erythromycin per ml in BHI agar</td>
</tr>
<tr>
<td>purC193</td>
<td>Pur&lt;sup&gt;−&lt;/sup&gt; purine requirement</td>
<td>-purines from CDS agar</td>
</tr>
<tr>
<td>rib-127</td>
<td>Rib&lt;sup&gt;−&lt;/sup&gt; riboflavin requirement</td>
<td>-riboflavin from CDS agar</td>
</tr>
</tbody>
</table>

<sup>a</sup>The concentration in parentheses was used to score unselected markers among recombinants.
agar (Difco Bacto), 25 g; sodium citrate $3\text{H}_2\text{O}$, 0.5 g; starch, 3.0 g; and sufficient deionized water to yield 1 liter of medium. DNase I was added to R medium by surface spreading 0.05 ml (3 mg/ml) volumes per plate just before use. R medium plates, which contained about 25 ml/15 x 100mm plate, were dried overnight at 35 C.

Cells harvested for DNA extraction were washed in Tris-EDTA-NaCl buffer (0.1M TRIS(hydroxymethyl)aminomethane, 0.15M NaCl, and 0.1M ethylenediamine-tetraacetic acid, pH 7.5). DNase-free ribonuclease-A (0.2%, type 1-A, Sigma) was dissolved in 0.15M NaCl, pH 5, heated in boiling water for 10 min, cooled, and neutralized to pH 7.0. Pronase solution was prepared by dissolving Pronase (Calbiochem) at 10 mg/ml in deionized water, adjusting the pH to 5.0, and heating to 80 C for 10 min. After cooling, the pH was adjusted to 7.0 and enough NaCl was added to reach a final concentration of 1 M. In later experiments, Protease type VI (Sigma) was used in place of Pronase. The RNase and Pronase solutions were dispensed into aliquots and stored at -20 C. SDS-ethanol solution contained 5 g sodium dodecyl sulfate, 50 ml 95% ethanol and 50 ml deionized water. Phenol was distilled under a nitrogen atmosphere and saturated with 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8.1, both on the day of use. Standard saline citrate (SSC), pH 7.2, contained
6.1 g NaCl, 4.4 g trisodium citrate and 100 ml deionized water. Tris-maleate buffer consisted of 0.1 M mono-tris (hydroxymethyl)aminomethane maleate (Trizma Maleate, Sigma) in deionized water at pH 7.0.

Protoplast Fusion Analysis

Fusion procedure

Parental cells harvested in saline (0.85% NaCl) from overnight BHI agar slants were inoculated into 100-ml volumes of TSB in 300-ml nephelometer flasks (Bellco) to an optical density (OD-540 nm) of 0.1. The cultures were shaken at 35 C in a reciprocating water bath (100 rpm; 3.8-cm cycles) until an OD of 0.65 (late-log cells) was reached. Because the parental strains exhibited different growth rates, each strain was inoculated into TSB at such a time that the cells from both cultures could be harvested simultaneously at the desired OD. The cells were harvested by centrifugation (10,000 x g, 25 min, 4 C) and washed once in saline. The cells from 200 ml of TSB from each parent strain were suspended in 10 ml of SMTB containing DNase (15 ug/ml) and lyso- staphin (30 ug/ml) and transferred to 50-ml screw-capped erlenmeyer flasks. The flasks were then gently turned for 45 min at 35 C on a rotary mixer with a horizontal shaft of rotation (36 rpm, 6-cm radius). The protoplasts were
harvested by centrifugation (3,400 x g, 10 min, 25 C) and each pellet was suspended gently in 1 ml of SMTB containing DNase (15 µg/ml). To a mixture containing 0.1 ml of each parent protoplast suspension was added 1.8 ml of 60% PEG 400 in SMTB. The fusion mixture then was gently but thoroughly mixed and incubated stationary in a circulating water bath at 20 C for 1 min. Aliquots (0.05 ml) of the fusion mixture were spread gently with glass spreaders on R medium agar plates. The plates were incubated at 35 C (65-80% relative humidity) for 7 days.

**Donor-recipient fusions**

When appropriate, a unidirectional transfer of genetic information was simulated with the following modifications of the fusion protocol. 1) The cells of both parents were grown to late-log phase (OD at 540 nm = 0.65-0.70), harvested and then washed 2X in saline. 2) The pelleted cells of the multiply-marked donor strain were suspended in 2 ml of saline and sonicated for 45 sec at a probe intensity of 50 (20 Kcps, Biosonik II sonicator, Bronwill Scientific, Rochester, NY) to disperse cell aggregates. The volume of the cell suspension was brought up to 10 ml and exposed to ultraviolet light (9.5 ergs/mm²/sec) for 30 sec with constant agitation in a 15 x 100 mm petri dish bottom. This UV dose resulted in a 3-log loss of viability. The irradiated donor cells were harvested
by centrifugation (3,400 x g, 10 min, 25 C) and both donor and recipient cells were prepared for protoplasting by suspending each in SMTB with DNase and lysostaphin (10 ml SMTB per 100 ml of original TSB culture). 3) One μg of erythromycin was added per ml of R medium for selection against the survival of the donor strain. All experimental steps following UV irradiation of the donor strain, including incubation of R medium plates, were performed under reduced illumination.

Genetic analysis

The growth on 5 R medium plates was collected in saline (5 ml/plate) and each suspension was sonicated for 1 min at a probe intensity of 50 (20 Kcps, Biosonik II sonicator) to disperse cell aggregates. After pooling the cell suspensions, diluted samples were spread on BHI + 1.5% agar to assay colony-forming units. Samples of the diluted and undiluted suspensions were also spread onto media of the desired selective properties. After incubation at 35 C for 48 h, approximately 600 isolated colonies from each selected class were picked with sterile toothpicks to the same selective medium to form master plates, each with an ordered array of 56 recombinants. After a maximum of 24 h at 35 C, the master plates were velveteen-replicated onto a medium of identical composition. After another 24 h incubation at 35 C, the replicas were velveteened to other selective media to score the distribution
of unselected markers among the recombinants. The scoring media were selective only for the single marker each was prepared to differentiate. From the master plates, the scoring plates were replicated in the following marker sequence (see Table 3 for medium composition); Isoleucine-valine, threonine, tryptophan, tyrosine, uracil, tetracycline, erythromycin, novobiocin, and methicillin. After 24 h incubation at 35 C, the replicas were examined and those plates of markers which had clearly differentiated into growth responses were prescored. After 48 h, the remaining plates were prescored; all plates, selective for the same marker, were arranged in a sequential order, in stacks, identical to the labeled sequence of the master plates. Contaminants and colonies missing in the ordered array of 56 per plate were clearly marked. From the stacks of plates, the growth responses were entered into the computer (CMB model 8032 computer, model 8050 dual disk drive, Commodore, Inc.) according to program instructions (the programs are listed in the Appendix). The data were loaded into a 2-dimensional matrix which ultimately contained each recombinant phenotype. From this point, the data were condensed and sorted into another array which contained all phenotypes observed in the fusion cross and the frequency at which each occurred. From these data, further manipulations were made until the marker-marker coinheritance
frequency matrix was assembled.

Controls

The controls incorporated into each experiment included the following modifications of the fusion procedure:

1) Parent cells received SMTB instead of lysostaphin during the preparation of protoplasts, so that normal cells of each parent were submitted to the fusion procedure - referred to as the Cell Control.

2) Substituting SMTB for the PEG solution during the fusion procedure, so that only spontaneous fusion events would be observed - referred to as the Fusion Control.

3) Adding PEG solution to a double volume of protoplasts from a single parent (2 of these controls were required per experiment) - referred to as the Reversion Controls.

High Molecular Weight DNA Purification

For each strain from which high molecular weight DNA was to be extracted, a 200-ml overnight TSB culture, incubated at 35 C in a reciprocating water bath shaker, was prepared. The cells were harvested by centrifugation (10,000 x g, 25 min, 4 C) and washed once in 10 ml of saline. After suspending the cells in 10 ml of SMTB, and
transferring them to a 50-ml screw-capped erlenmeyer flask, 0.3 ml of lysostaphin stock solution was added. Protoplasts were generated as described previously in the protoplast fusion protocol. The protoplasts were harvested by centrifugation (3,400 x g, 10 min, 25 C) and the supernatant decanted. The pelleted protoplasts were partially lysed by adding 5 ml of Tris-EDTA-NaCl buffer and further lysis was promoted by gently scraping the pellet from the tube bottom with the end of a 1-ml pipet. The partially lysed protoplast mixture was poured into a 50-ml screw-capped erlenmeyer flask and 0.5 ml of DNase-free RNase was added. After swirling into solution, the mixture was incubated stagnant at 35 C for 60 min. After adding 1 ml of Pronase solution, the incubation was continued for another 60 min. SDS-ethanol (0.6 ml) was introduced and the mixture was hand shaken vigorously for 1 min (vertically at 2 cycles/sec). Six milliliters of tris-saturated phenol was added and the lysate was swirled until an emulsion formed. The mixing was continued on a roller extractor (horizontal axis of rotation, 20 rpm) for 5 min. The phases were separated by centrifugation (10,000 x g, 60 min, 4 C) and the upper aqueous phase was carefully removed and transferred to a 50-ml screw-capped erlenmeyer flask. Two volumes of cold (4 C) 95% ethanol were added and the DNA precipitate
was spooled onto a glass rod. After washing in fresh cold ethanol the precipitate was held overnight at 4 C in ethanol. Within 24 hr the precipitate was aseptically transferred to a test tube containing sterile SSC (4 ml) and was stored at 4 C for at least 72 hr before sterility or genetic activity was assessed.

Standard Protocol for DNA Purification

Standard transforming DNA was prepared according to a modification (Pattee and Neveln, 1975) of the procedure of Lindberg et al. (1972). A 100-ml volume of BHI was inoculated with the strain from which the DNA was to be extracted, and incubated overnight with shaking at 35 C. The cells were harvested (10,000 x g, 30 min, 4 C), washed once with Tris-EDTA-NaCl buffer (20 ml), suspended in 5 ml of the same buffer, and transferred to a 50-ml screw-capped erlenmeyer flask. Lysostaphin (0.3 ml) was added and the mixture was incubated with shaking (110 cycles/min, 3.8-cm strokes) for 30 min at 35 C. Pronase (1 ml) was added and incubation, with shaking, was continued for 5 min, followed by incubation without shaking for 55 min. Then 0.6 ml of SDS-ethanol was added and the flask was shaken vigorously on a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, Pa.) for 30 min at room temperature. The lysis mixture was then
extracted twice with equal volumes of phenol saturated with Tris buffer. After each phenol extraction (horizontal axis of rotation, 60 rpm for 30 min), the emulsion was separated into phenolic and aqueous phases by centrifugation (10,000 x g, 60 min, 4 C) and the aqueous phase removed. The aqueous phase from the second extraction was washed with 20 ml of ethyl ether, and the residual ether was removed under a stream of nitrogen gas. The remaining steps, including ethanol precipitation and washing, were performed as described in the protocol for high molecular weight DNA extraction.

Transformation: Artificially Induced Competence

Competent cells for transformation were prepared by a modification (Pattee and Neveln, 1975) of the procedure described by Lindberg et al. (1972). To insure maximum frequencies of transformation, normal rabbit serum (Pel-Freez Biologicals, Rogers, Arizona) and phage 55 lysates were used to supplement the natural competence activity of the recipient cells (Thompson and Pattee, 1977). Cells from an overnight BHI agar slant culture of the recipient strain were suspended in 5 ml of saline. A sufficient amount of this suspension was used to inoculate a 100-ml volume of TSB in a 300-ml nephelometer flask to reach an optical
density of 0.2 (540 nm). Ten milliliter-volumes of this suspension were used to inoculate 100-ml volumes of TSB in 300-ml nephelometer flasks, and the cultures were incubated in a reciprocating water bath shaker (110 cycles/min, 3.8-cm strokes, 35 C). At an OD of about 0.15-0.20 the cells were harvested (10,000 x g, 30 min, 4 C) and suspended and pooled in TSB (1 ml TSB/300 ml starting culture). Normal rabbit serum (0.5 ml serum/300 ml starting culture) and phage 55 lysate (propagated on S. aureus Ps 55, 1-2 x 10^{11} pfu/ml in TBS; 1 ml added/300 ml starting culture) were added to the cell suspension. After a 5-min incubation with shaking at 35 C the cells were harvested and washed in cold Tris-maleate buffer and finally resuspended in the same buffer containing 0.1 M CaC\(_{11}\) (1 ml buffer/100 ml starting culture). One milliliter-volumes of the cell suspension were immediately transferred to 15-ml corex centrifuge tubes containing 0.15-ml portions of the appropriate DNA or to empty tubes (cell controls). The tubes were gently mixed and then held in an ice bath for 2.5 min, followed by an incubation, with shaking, for 2.5 min at 35 C. The cells were then harvested and each pellet was suspended in 1 ml of BHI and incubated with shaking at 35 C for 60 min. This suspension was harvested and each pellet was suspended in 1 ml of saline. Aliquots (0.1 ml)
of this suspension, with or without dilution, were spread on the appropriate selective media to assay for transformants or revertants. Viable cell counts were obtained by plating diluted samples on BHI agar plates. The segregation of unselected markers was scored by velveteen-replicating the master plates containing the recombinant clones onto media of the appropriate composition (see Table 3).
RESULTS

In several preliminary protoplast fusion experiments involving different pairs of parent strains, recombination was measured by velveteening the growth directly from R medium onto CDS agar selective for combinations of parental markers. Recombinants possessing phenotypic characteristics derived from both parents were readily recovered by this technique; however, when examined for homogeneity of parental markers, these recombinant clones were often observed to be heterogeneous for unselected markers involved in the cross (data not shown). While useful for some purposes, this method was judged to be inappropriate for accurately measuring the frequency of genetic recombination leading to stable haploid recombinants.

To facilitate the recovery of classes of recombinants possessing specific markers from each parent that were also homogeneous for unselected markers, subsequent experiments were performed by harvesting the growth from R medium, and respreading the cells on selective media after disaggregation by sonication. The validity of this technique was confirmed as follows. Protoplasts of strains ISP193 and ISP483 were fused, allowed to regenerate on R medium, and the appropriate dilutions of the sonicated cells spread on CDS agars selective for Thy^{+}His^{+} and Thy^{+}His^{+}Em^{R} recombinants.
Six clones from each selective medium were struck for isolation on CDS agar of identical composition, and 25 colonies from each streak plate were then picked and scored for unselected parental markers. While the distribution of unselected markers differed from one clone to another, the colonies derived from each clone were always identical.

Optimum Conditions for Protoplast Fusion

To define the optimum conditions for protoplast fusion, and to facilitate comparing the results from different experiments, strains ISP193 and ISP483 were used as parents in a series of fusion experiments. Selection was always for Thy^His^ and Thy^His^Em^ recombinants, and in some experiments, unselected markers were scored among the recombinants.

To determine if culture age contributed to differences in recombination frequencies (expressed as numbers of recombinants/1 x 10^9 colony forming units (CFU) harvested from R medium), protoplasts were prepared from strains ISP193 and ISP483 harvested from TSB at various stages of growth; these protoplasts were fused, spread on R medium, and the resulting CFU harvested and assayed for Thy^His^ and Thy^His^Em^ recombinants. The results (Table 4) demonstrated that cells harvested from log-phase cultures gave higher recombination frequencies than those harvested from TSB at
Table 4. The effect of parent culture age on recombination frequencies following protoplast fusion

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Recombination frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recombination frequency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; His&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; His&lt;sup&gt;+&lt;/sup&gt; Em&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Early-log (0.35-0.40)</td>
<td>$1.1 \times 10^6$</td>
<td>$3.9 \times 10^5$</td>
</tr>
<tr>
<td>Mid-log (0.45-0.50)</td>
<td>$1.0 \times 10^6$</td>
<td>$7.1 \times 10^5$</td>
</tr>
<tr>
<td>Late-log (0.65-0.70)</td>
<td>$2.7 \times 10^6$</td>
<td>$6.4 \times 10^5$</td>
</tr>
<tr>
<td>Early stationary (1.0)</td>
<td>$2.6 \times 10^6$</td>
<td>$5.7 \times 10^4$</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of Thy<sup>+</sup> His<sup>+</sup> or Thy<sup>+</sup> His<sup>+</sup> Em<sup>+</sup> recombinants (ISP193 x ISP483 cross) per $1 \times 10^9$ viable cells harvested from one regeneration plate of R medium.

<sup>b</sup>The growth of both ISP193 and ISP483 in TSB was monitored turbidimetrically at 540 nm; the figures in parentheses are approximate optical densities (540 nm) at which cells were harvested.

early stationary phase of growth.

Protoplasts prepared from cells by treatment with lysostaphin for variable time periods exhibited differences in recombination frequencies (Table 5). The optimum exposure time to lysostaphin appeared to be 45 min, while exposure for 120 min significantly reduced recombination frequencies. Moreover, prolonged exposures (120 min) resulted in a marked reduction in turbidity along with the formation of a flaky
Table 5. Effect of time of exposure of parent cells to lysostaphin on recombination frequencies

<table>
<thead>
<tr>
<th>Duration of exposure to lysostaphin (min.)</th>
<th>Recombination frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Thy&lt;sup&gt;+&lt;/sup&gt;His&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Thy&lt;sup&gt;+&lt;/sup&gt;His&lt;sup&gt;+&lt;/sup&gt;Em&lt;sup&gt;R&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>6.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>9.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4.7 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>1.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>7.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of Thy<sup>+</sup>His<sup>+</sup> or Thy<sup>+</sup>His<sup>+</sup>Em<sup>R</sup> recombinants (ISP193 x ISP483 cross) per 1 x 10<sup>9</sup> viable cells harvested from one regeneration plate of R medium.

precipitate, suggestive of a reduced recovery of viable protoplasts. When cells were incubated with lysostaphin for less than 30 min, their conversion to protoplasts was inadequate for experimental purposes. Under these conditions, prohibitively large numbers of nonprotoplasted cells remained in the protoplast preparations. These walled cells were detected by spreading samples on BHI agar lacking sucrose (where only walled cells yield colonies); they also gave rise to more rapid growth into colonies on R medium. These early-forming colonies interfered with fusion experiments because they always were surrounded by a zone within which regeneration of protoplasts was inhibited. When
examining the effect of agar concentration in R medium on recombination frequencies, it was observed that 1% and 2.5% agar resulted in equivalent frequencies of recombination (defined as recombinants/1 x 10^9 CFU). However, 2.5% agar in R medium resulted in an estimated 10-fold increase in the numbers of colonies recovered on each plate; a major factor in this increase was a striking reduction in the diameter of the zone of inhibition surrounding the early-forming colonies.

Significant differences in recombination frequencies were observed when PEG molecular weight and concentration were varied. The source of all chemical stocks of PEG was Sigma Chemical Company, St. Louis, Mo. High recombination frequencies were induced with 60% PEG 1000 (Table 6). When the PEG stock solution contained 15% (v/v) dimethyl sulfoxide (DMSO), a significant increase in recombination was observed. Concentrations of PEG 1000 greater than 60% were difficult to work with because of high viscosity and a tendency to solidify at ice bath temperatures (8-10°C). Solutions of PEG 400 and PEG 200, however, could be manipulated at concentrations up to 100% at 8-10°C. PEG 400 induced high, but fluctuative, recombination frequencies at concentrations from 50 to 100% (Table 7). Fusions with 60% PEG 400 + 15% DMSO induced high recombination frequencies but fusions
Table 6. The effect of different combinations of PEG 1000 on recombination frequencies

<table>
<thead>
<tr>
<th>PEG stock concentration</th>
<th>DMSO</th>
<th>Recombination frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt;His&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt;His&lt;sup&gt;+&lt;/sup&gt;Em&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%</td>
<td>-</td>
<td>6.0 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>6.0 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
<td>2.7 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>9.0 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>30%</td>
<td>-</td>
<td>1.2 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.4 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>40%</td>
<td>-</td>
<td>4.3 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>50%</td>
<td>-</td>
<td>7.5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>50%</td>
<td>+</td>
<td>5.6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>60%</td>
<td>-</td>
<td>9.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.9 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>60%</td>
<td>+</td>
<td>7.3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of Thy<sup>+</sup>His<sup>+</sup> or Thy<sup>+</sup>His<sup>+</sup>Em<sup>r</sup> recombinants (ISP193 x ISP483 cross) per 1 x 10<sup>9</sup> viable cells harvested from one regeneration plate of R medium.

<sup>b</sup>Polyethylene glycol (PEG 1000; m.w. = 1000) stock concentration (v/v in SMTB).

<sup>c</sup>A + refers to the addition of dimethyl sulfoxide to a final concentration of 15% (v/v) to the PEG stock solution.
Table 7. The effect of different concentrations of PEG 400 on recombination frequencies

<table>
<thead>
<tr>
<th>PEG stock concentration</th>
<th>DMSO</th>
<th>Recombination frequencya</th>
<th>Thy\textsuperscript{+}His\textsuperscript{+}</th>
<th>Thy\textsuperscript{+}His\textsuperscript{+}Em\textsuperscript{r}</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>-</td>
<td>4.8 x 10\textsuperscript{1}</td>
<td>4.8 x 10\textsuperscript{1}</td>
<td></td>
</tr>
<tr>
<td>30%</td>
<td>-</td>
<td>4.0 x 10\textsuperscript{1}</td>
<td>4.0 x 10\textsuperscript{1}</td>
<td></td>
</tr>
<tr>
<td>40%</td>
<td>-</td>
<td>5.0 x 10\textsuperscript{5}</td>
<td>2.6 x 10\textsuperscript{5}</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>-</td>
<td>5.7 x 10\textsuperscript{6}</td>
<td>7.0 x 10\textsuperscript{5}</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>+</td>
<td>7.7 x 10\textsuperscript{6}</td>
<td>2.8 x 10\textsuperscript{6}</td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>-</td>
<td>1.4 x 10\textsuperscript{6}</td>
<td>4.7 x 10\textsuperscript{5}</td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>+</td>
<td>1.1 x 10\textsuperscript{7}</td>
<td>4.6 x 10\textsuperscript{6}</td>
<td></td>
</tr>
<tr>
<td>70%</td>
<td>-</td>
<td>9.5 x 10\textsuperscript{6}</td>
<td>2.3 x 10\textsuperscript{6}</td>
<td></td>
</tr>
<tr>
<td>70%</td>
<td>+</td>
<td>NT\textsuperscript{d}</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>-</td>
<td>2.7 x 10\textsuperscript{7}</td>
<td>6.4 x 10\textsuperscript{5}</td>
<td></td>
</tr>
<tr>
<td>90%</td>
<td>-</td>
<td>1.2 x 10\textsuperscript{7}</td>
<td>2.7 x 10\textsuperscript{5}</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>-</td>
<td>1.9 x 10\textsuperscript{7}</td>
<td>3.5 x 10\textsuperscript{6}</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Expressed as the number of Thy\textsuperscript{+}His\textsuperscript{+} or Thy\textsuperscript{+}His\textsuperscript{+}Em\textsuperscript{r} recombinants (ISP193 x ISP483 cross) per 1 x 10\textsuperscript{9} viable cells harvested from one regeneration plate of R medium.

\textsuperscript{b}Polyethylene glycol (PEG 400; \textsc{m.w.} = 400) stock concentration (v/v in SMTB).

\textsuperscript{c}A + refers to the addition of dimethyl sulfoxide to a final concentration of 15% (v/v) to the PEG stock solution.

\textsuperscript{d}Not tested (growth and regeneration on R medium was inhibited or slowed).
with 70% PEG 400 + 15% DMSO resulted in inhibited regeneration on R medium. High concentrations of PEG 200 (80, 90, and 100%) also inhibited protoplast regeneration, as did 70% PEG 200 + 15% DMSO (Table 8).

Table 8. The effect of different concentrations of PEG 200 on recombination frequencies

<table>
<thead>
<tr>
<th>PEG stock concentration</th>
<th>DMSO</th>
<th>Recombination frequency $^{a}$</th>
<th>Recombination frequency $^{a}$</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>$^{+}$ ThyHis $^{+}$</td>
<td>ThyHis $^{+}$Em $^{r}$</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
<td>$1.6 \times 10^{2}$</td>
<td>$1.8 \times 10^{1}$</td>
</tr>
<tr>
<td>30%</td>
<td>-</td>
<td>$1.4 \times 10^{1}$</td>
<td>$1.4 \times 10^{1}$</td>
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<td>-</td>
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<td>$4.1 \times 10^{4}$</td>
</tr>
<tr>
<td>50%</td>
<td>-</td>
<td>$1.0 \times 10^{5}$</td>
<td>$5.9 \times 10^{3}$</td>
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<tr>
<td>50%</td>
<td>+</td>
<td>$1.4 \times 10^{6}$</td>
<td>$1.0 \times 10^{5}$</td>
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<tr>
<td>60%</td>
<td>-</td>
<td>$4.1 \times 10^{5}$</td>
<td>$3.6 \times 10^{5}$</td>
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<tr>
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<td>$2.7 \times 10^{5}$</td>
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<tr>
<td>70%</td>
<td>-</td>
<td>$3.2 \times 10^{6}$</td>
<td>$1.1 \times 10^{6}$</td>
</tr>
<tr>
<td>70%</td>
<td>+</td>
<td>NT $^{d}$</td>
<td>NT $^{d}$</td>
</tr>
</tbody>
</table>

$^{a}$Expressed as the number of Thy$^{+}$His$^{+}$ or Thy$^{+}$His$^{+}$Em$^{r}$ recombinants (ISP193 x ISP483 cross) per $1 \times 10^{9}$ viable cells harvested from one regeneration plate of R medium.

$^{b}$Polyethylene glycol (PEG 200; m.w. = 200) stock solution (v/v in SMTB) concentration. High concentrations of PEG 200 (80, 90, 100%) inhibited growth and regeneration on R medium.

$^{c}$A + refers to the addition of dimethyl sulfoxide to a final concentration of 15% (v/v) in the PEG stock solution.

$^{d}$Not tested (growth and regeneration on R medium was inhibited or slowed).
An attempt was made to increase the incidence of protoplast fusion by increasing the temperature at which fusion mixtures (PEG stock solution + parent protoplast suspensions) were incubated. An increase in the incidence of protoplast fusion was assumed to be reflected in an increase in recombination frequency. As shown in Table 9, fusions performed at 20°C resulted in higher recombination frequencies when compared to those done at 8-10°C (the temperature used for the preceding optimization experiments). Fusions done at 30°C resulted in a marked decrease in regenerative abilities of the protoplasts on R medium.

Table 9. The effect of temperature at which fusion was done on recombination frequencies

<table>
<thead>
<tr>
<th>Fusion temperature</th>
<th>Recombination frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; His&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; His&lt;sup&gt;+&lt;/sup&gt; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>8°C</td>
<td>4.5 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>20°C</td>
<td>1.3 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>4.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of Thy<sup>+</sup> His<sup>+</sup> or Thy<sup>+</sup> His<sup>+</sup> Em<sup>r</sup> recombinants (ISP193 x ISP483 cross) per 1 x 10<sup>9</sup> viable cells harvested from one regeneration plate of R medium.

<sup>b</sup>The fusion mixture contained the parent protoplasts and 1.8 ml 60% PEG 400 plus 15% DMSO.

<sup>c</sup>Not tested (growth and regeneration was inhibited on R medium).
Some nutritional aspects of protoplast regeneration and growth on R medium were also examined. Both shortened regeneration time (incubation time on R medium required for good growth) and increased regeneration frequencies were considered as desirable results. When yeast extract (0.1%, Difco) or sodium pyruvate (1%, Sigma), or both, were added to R medium, reduced recombination frequencies resulted (data not shown). Significantly reduced regeneration and growth was observed when BHI was substituted for TSB in R medium.

Optimum regeneration was observed when the R medium plates were incubated (35 C) at lower relative humidities (20-50%) until the inoculum-DNase mixture had completely adsorbed into the medium. To prevent further drying during the subsequent prolonged incubation, the plates were placed in a humidified incubator (approximately 75% relative humidity, 35 C).

Some additional variables that influenced the efficiency of regeneration were observed. R medium plates could be predried overnight at 35 C and, after being inoculated with the fused protoplasts, placed directly into a humidified environment (approximately 80% relative humidity) for the duration of the incubation.

One of the most striking changes made was the higher
regeneration frequencies when R medium was supplemented with 0.3% potato starch (Sigma). Protoplasts were prepared from ISP933 and ISP800 and fused as described in the Materials and Methods. From Petroff-Hausser chamber counts of protoplast suspensions, an estimated $2 \times 10^9$ protoplasts per ml were contained in the fusion mixture (PEG stock solution plus parent protoplasts). By diluting and plating the fusion mixtures onto R medium plates, a 10-fold increase in regeneration frequency, (1% as compared to 0.1%), was observed when R medium was supplemented with 0.3% starch. Regeneration frequency was defined as the viable counts on R medium divided by the estimate of the total protoplasts per ml in the fusion mixture x 100. R medium plates containing the most concentrated platings of the fusion mixture were velveteen-replicated to selective media. An approximate 10-fold increase in the numbers of recombinant foci appeared on the replicas from starch containing R medium plates.

Genetic Analysis by Protoplast Fusion

Experimental reproducibility

Many of the decisions regarding changes in the experimental protocol described above were based on differences in recombination frequencies. Since the major interest in protoplast fusion was its use for chromosome mapping,
reproducibility of the data was important. Protoplasts of strains ISP193 and ISP483 were fused, allowed to regenerate on R medium, and the appropriate dilutions of the sonicated cells (pooled from the saline harvests of 5 R medium plates) were spread on CDS media selective for Thy^+His^+ and Thy^+His^+Em^r recombinants. Approximately 200-300 recombinants of each class were scored for unselected markers. This experiment was repeated four times and recombination frequencies for each selection and the distribution of unselected markers was compared using simple statistical analyses (Table 10). The estimates for recombination frequencies of both selections were significantly variable (plus or minus 50%), but estimates for the distribution of Ilv^- and Thr^- phenotypes among the recombinants was relatively stable.

Construction of isogenic strains

Probably one of the most significant aspects of developing a fusion analysis system in *S. aureus* was the construction of isogenic strains for use as parents in 9- or 10-factor genetic crosses. Isogenic strains were constructed to minimize the possibility that differences in chromosome homology would contribute to erroneous linkage data. As a result, most protoplast fusion experiments were crosses between derivatives of *S. aureus* 8325 that exhibited the same phage typing pattern. Two distinct classes of
Table 10. Experimental reproducibility of recombination frequencies and distribution of unselected markers among selected classes

<table>
<thead>
<tr>
<th>Selected class</th>
<th>Recombination frequency(^b)</th>
<th>Percentage that were:(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy(^+)His(^+)</td>
<td>3.6 \times 10^6 (44%)</td>
<td>49.5% (2.3) 49.2% (2.3)</td>
</tr>
<tr>
<td>Thy(^+)His(^+)Em(^r)</td>
<td>5.8 \times 10^5 (52%)</td>
<td>84.5% (4.4) 43.2% (4.6)</td>
</tr>
</tbody>
</table>

\(^a\)Mean results from 4 fusion experiments (ISP193 x ISP483). The numbers within parentheses are standard deviation values.

\(^b\)Expressed as the number of Thy\(^+\)His\(^+\) or Thy\(^+\)His\(^+\)Em\(^r\) recombinants per 1 \times 10^9 total viable cells from a combined harvest of 5 regeneration medium plates. Mean results are from 4 fusion experiments (ISP193 x ISP483). The numbers within parentheses are percent variations from the mean value (standard deviation divided by the mean x 100).

Parent strains were constructed; a multiply-marked parent with 8 or 9 chromosomal markers, (a combination of antibiotic resistance determinants and determinants that confer additional nutritional requirements), and a parent which contained the marker of interest. The marker of interest was usually a chromosomal Tn551 insertion mutation, which prior to analysis by fusion with the multiply-marked parent, was transformed into the ISP794 genetic background. Some of the Tn551 insertion mutations designated for use in fusion analysis had been mapped by transformation and
occupied chromosomal sites of interest, such as the known ends of the linkage groups. Other Tn551 insertions transformed into the ISP794 genetic background had been shown not to be within the known linkage groups and were designated as orphan insertions.

A strain was needed that possessed a sufficient number of markers so that when crossed with a parent containing a Tn551 insertion mutation, a close linkage of the insertion with markers in the multiply-marked parent would not be overlooked. The original multiply-marked parent strain (ISP769) was constructed by fusing ISP483 (irradiated to affect a 3-log kill before use) and ISP661. After regeneration on R medium, selection was made for the inheritance of the Nov^ determinant into ISP661. ISP769 was one of a few recombinants which inherited all markers from both parents, and possessed the same phage-typing pattern as ISP794. ISP769 contains lys-115, trp-103, and thrB106 in linkage group I, ilv-129 in linkage group III, and uraAl41, hisG15, nov-142, and meo-4916 in linkage group II. The tmn-3106 and ala-126 markers were introduced into ISP769 by transformation with ISP268 DNA, resulting in ISP845. Crossovers were found to be rare between hisG15 and nov-142 (less than 0.1% of the total recombinants) when ISP845 was used as the multiply-marked parent in fusion analysis. Consequently, ISP852 was made by repairing hisG15 in ISP845 with ISP2 DNA.
After several fusion experiments with ISP852 as the multiply-marked parent, it was found that among the recombinants, the Lys phenotype could not be scored (as growth or no growth) reliably on CDS medium deficient in lysine. ISP933 was constructed through a series of transformations (see Table 1) which ultimately replaced lys-115 with tyrB282::Tn551 ermB321. This determinant occupied a more "strategic" location on the extreme end of linkage group I. Fusion analyses with ISP933 as a parent resulted in approximately 99% of the recombinants being Ala⁺, regardless of what selected markers were used. The multiply-marked strains used for the majority of the fusions reported here were both derived by repairing ala-126 in ISP933. ISP983 was constructed by introducing the silent insertion, Ω5[Chr::Tn551], and ala⁺ into ISP933 by transformation with ISP196 DNA. ISP988 was constructed by transforming ISP983 with ISP267 DNA; it is a Tmn⁺ transformant that had lost Ω5[Chr::Tn551].

Two other markers were found to behave unacceptably in fusion analyses; thy-101 and pig⁺ were expressed among fusion recombinants at very low frequencies (less than 0.1% regardless of selected marker choices). Therefore, all subsequent experiments were done with parents that always were Thy⁺ and Pig⁻.
Fusion controls

When selecting for Thy\textsuperscript{+}His\textsuperscript{+} or Thy\textsuperscript{+}His\textsuperscript{+}Em\textsuperscript{r} recombinants in an ISP193 x ISP483 fusion, 1 to 5 x 10\textsuperscript{0} recombinants per 1 x 10\textsuperscript{9} CFU were harvested from regeneration plates of the Reversion Controls. These frequencies were comparable to normal reversion frequencies of the thy-101 and hisGl5 markers of the parental strains. However, in these same crosses, significantly higher recombination frequencies of 1 x 10\textsuperscript{1} to 1 x 10\textsuperscript{3} per 1 x 10\textsuperscript{9} CFU were observed when regeneration plates of the Cell Control and Fusion Control were harvested and plated. The phenotypes of these "recombinants" were examined and all appeared to be either Thy\textsuperscript{+} revertants of ISP193 or His\textsuperscript{+} revertants of ISP483. In fusions performed between isogenic parents, these anomalies were not observed. The control frequencies were from 1 x 10\textsuperscript{0} to 5 x 10\textsuperscript{1} per 1 x 10\textsuperscript{9} CFU and each frequency correlated with the normal spontaneous mutation rate of the selected marker involved in the cross.

Simulated unidirectional exchange of genetic information

The unique characteristics of protoplast fusion as a mode of genetic exchange and recombination in procaryotes are that genetic exchange is bidirectional and intact chromosomes are combined in the same cytoplasm at high frequencies. Many early experiments directed at genetic
mapping of chromosomal markers by protoplast fusion made use of selection for 3 or more markers, some of which were nutritional. Perhaps because of physiological stress, selection for many combinations of nutritional and antibiotic resistance markers resulted in poor or nonexistent colony formation on CDS media. For this reason, the procedure for analysis of recombinants required that a pair of antibiotic resistance determinants (one from each parent) be selected. Selection exclusively for antibiotic resistance determinants was most consistent, as background growth (consisting of parental and unselected recombinant phenotypes) was suppressed adequately by the presence of antibiotics in the medium. In some nutritional selections, undesired recombinant or parental phenotypes sometimes were carried over to the master plates in the transfer process. As a result, scoring for the distribution of unselected markers was complicated by the fact that many of the "clones" on the master plates were not pure cultures of the desired recombinant phenotype.

We attempted to simplify fusion analysis by experimentally simulating a unidirectional transfer of genetic information. Three experimental parameters established this relationship: 1) The multiply-marked erythromycin-sensitive parent (designated the donor) regenerated from protoplasts at low efficiency
on R medium. 2) A three-log kill of ultraviolet radiation was administered to the donor strain before protoplast formation. 3) The survival of the donor protoplasts was selected against by adding erythromycin (1 μg/ml) to R medium. The recipient strain carried a Tn551 insertion mutation; therefore, differences in the results from one experiment to another depended solely on the map location of the transposable element in the recipient strain.

**Fusion analysis of the Ω1028[Chr::Tn551] locus** From interpretations of the results of an ISP852 (donor) x ISP810 (recipient) protoplast fusion, the relative map location of Ω1028[Chr::Tn551], an orphan insertion, was predicted. Selection was made for Em^Tmn^, and the phenotypes of 616 recombinants were sorted for analysis as marker-marker coinheritance frequencies were generated by a programmed computer. The analysis produced coinheritance frequencies for all possible pairs of markers in the form of a 2-dimensional matrix. The coinheritance frequency (CIF) for a specific pair of markers was defined as the percentage of the total number of recombinants that exhibited either parental phenotype for that specific pair of markers. Since crossovers between closely linked markers occur at lower frequencies than between distantly linked markers, closely linked markers should coinherit at high frequencies.
The data in Table 11 confirmed these predictions. High co-inheritance frequencies were observed between markers which were known to be linked by previous transformation analyses. In linkage group I, ThrB and Trp coinherited 89% of the time. The Tmn marker in linkage group III coinherited with Ala in 92% of the recombinants and with Ilv in 73% of the recombinants. All three markers in linkage group II, (UraA, Nov, and Mec) exhibited CIF values greater than 90%.

Since the insertion mutation coinherited with ThrB 94% of the time and with Trp 84% of the time, its relative map location was predicted to be "left" of the thrB106 - trp-103 markers in linkage group I. Another unique linkage was apparent as Ilv, an end marker of linkage group III, and UraA, an end marker of linkage group II, exhibited a CIF value of 81%.

From the same cell suspension obtained from the ISP852 x ISP810 cross, dilutions were made onto media selective for Nov⁰Em⁰ recombinants. These data are also presented in Table 11 and they confirmed the predictions made from the Em⁰Tmn⁰ recombinant analysis, with the exception of the ilv-129 uraA141 linkage.

The predicted position of Ω1028[Tn]:Tn551 by fusion analysis was confirmed by transforming ISP926 with DNA (purified with the standard protocol) from ISP810 (Table 12).
Table 11. Coinheritance frequencies for an ISP852 x ISP810\textsuperscript{a} protoplast fusion\textsuperscript{b}

<table>
<thead>
<tr>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
<th>Ala</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Em</td>
<td>100</td>
<td>94</td>
<td>84</td>
<td>8</td>
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<td>36</td>
<td>34</td>
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<tr>
<td>Thr</td>
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<td>99</td>
<td>98</td>
<td>14</td>
<td>6</td>
<td>32</td>
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<td>36</td>
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<tr>
<td>Trp</td>
<td>84</td>
<td>89</td>
<td>100</td>
<td>19</td>
<td>16</td>
<td>33</td>
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<tr>
<td>Ala</td>
<td>8</td>
<td>14</td>
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<td>69</td>
<td>70</td>
<td>71</td>
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<tr>
<td>Tmn</td>
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<td>16</td>
<td>92</td>
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<td>73</td>
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<tr>
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<td>40</td>
<td>40</td>
<td>70</td>
<td>64</td>
<td>81</td>
<td>100</td>
<td>94</td>
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<tr>
<td>Nov</td>
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<td>36</td>
<td>37</td>
<td>71</td>
<td>65</td>
<td>78</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>Mec</td>
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<td>36</td>
<td>70</td>
<td>66</td>
<td>77</td>
<td>93</td>
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<table>
<thead>
<tr>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
<th>Ala</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
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<tbody>
<tr>
<td>Em</td>
<td>100</td>
<td>86</td>
<td>86</td>
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<td>53</td>
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<td>96</td>
<td>91</td>
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<tr>
<td>Ilv</td>
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<td>50</td>
<td>51</td>
<td>91</td>
<td>91</td>
<td>100</td>
<td>56</td>
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<tr>
<td>Ura</td>
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<td>24</td>
<td>55</td>
<td>56</td>
<td>56</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Nov</td>
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<td>14</td>
<td>14</td>
<td>45</td>
<td>47</td>
<td>47</td>
<td>90</td>
<td>100</td>
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<td>Mec</td>
<td>0</td>
<td>14</td>
<td>15</td>
<td>46</td>
<td>48</td>
<td>47</td>
<td>90</td>
<td>99</td>
</tr>
</tbody>
</table>

\textsuperscript{a}ISP852 = Em\textsuperscript{−}Thr\textsuperscript{−}Trp\textsuperscript{−}Ala\textsuperscript{−}Tmn\textsuperscript{−}Ilv\textsuperscript{−}Ura\textsuperscript{−}Nov\textsuperscript{−}Mec\textsuperscript{−}

\textsuperscript{b}ISP810 = Em\textsuperscript{+}Thr\textsuperscript{+}Trp\textsuperscript{+}Ala\textsuperscript{+}Tmn\textsuperscript{+}Ilv\textsuperscript{+}Ura\textsuperscript{+}Nov\textsuperscript{+}Mec\textsuperscript{+}

\textsuperscript{b}The donor strain was irradiated and was selected against by adding erythromycin to R medium. The recombination frequency for the Em\textsuperscript{Tmn\textsuperscript{−}} selection was 5.3 x 10\textsuperscript{5} per 1 x 10\textsuperscript{9} cfu, and 616 recombinants were scored. The recombination frequency for the Em\textsuperscript{Nov\textsuperscript{−}} selection was 2.1 x 10\textsuperscript{6} per 1 x 10\textsuperscript{9} cfu, and 448 recombinants were scored.

\textsuperscript{c}The numbers represent, for each pair of markers, the percentage of the total number of Em\textsuperscript{Tmn\textsuperscript{−}} or Em\textsuperscript{Nov\textsuperscript{−}} recombinants with either parent phenotype.
Table 12. Transformation of ISP926 with standard protocol DNA purified from ISP810

<table>
<thead>
<tr>
<th>Relevant phenotypes</th>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor: r + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient: s - -</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Of 406 Em\(^R\) transformants:
- 85.7\% (348) r - -
- 13.1\% (53)  r + -
- 1.2\% (5)    r + +

\(^a\)The frequency of transformation and the rate of spontaneous resistance was 300 per 1 x 10\(^9\) cfu and 30 per 1 x 10\(^9\) cfu, respectively.

Among the Em\(^R\) transformants, 13\% cotransformed with thrB106, 1\% cotransformed with both thrB106 and trp-103 and the Em\(^R\)Trp\(^+\) transformants were all Thr\(^+\). From these data, it can be concluded that \(\Omega\)1028[Chr::Tn551] maps to the left of thrB106 in linkage group I in the order \(\Omega\)1028 - thrB - trp.

Bidirectional exchange of genetic information by fusion

Many of the parameters used to establish a "unidirectional" transfer of information (donor-recipient fusions) were of concern because of their possible roles in creating anomalies in the data. The logical basis of coinheritance frequency measurements, as demonstrated in the ISP852 x ISP810 cross, should prevail if the exchange of genetic information is random and bidirectional. As a result of these considerations, the
Experimental parameters of fusion analysis were adjusted to make the exchange of information as random as possible. The irradiation step was eliminated, no selection was made during regeneration, and multiply-marked parents were used that regenerated efficiently on R medium (ISP933, ISP988, and ISP983). To evaluate the frequency of recombination under these conditions, an ISP933 x ISP808 fusion was performed. After regeneration (in the absence of selection), the cells were harvested from the R medium, sonicated, diluted, and inoculated onto BHI agar (devoid of selection). Twelve hundred of the resulting colonies then were analyzed for all markers involved in the cross. Of these 1200 colonies, 6% were recombinants. Among the parental classes, 75% were ISP808 phenotype and 25% were ISP933 phenotype. Thus, even under conditions designed to allow completely random genetic recombination and regeneration, the parental strain growth rates on R medium (and perhaps the efficiency of regeneration) apparently were influenced by genetic background.

**Fusion analysis of the Δ42[Chr::Tn551] locus** The relative map position of Δ42[Chr::Tn551], another orphan insertion, was predicted by examining CIF values generated from an ISP933 x ISP803 protoplast fusion. As seen in Table 13 (analysis of Em^rTmn^r and Em^rNov^r recombinants), Em exhibited high CIF values with ThrB and TrpE and a low CIF value with
Table 13. Coinheritance frequencies for an ISP933 x ISP803^a protoplast fusion^b

<table>
<thead>
<tr>
<th></th>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
<th>Tyr</th>
<th>Ala</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coinheritance frequencies^c for Em^Tmn^ recombinants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Em</td>
<td>100</td>
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<td>61</td>
<td>17</td>
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<td>84</td>
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<td>66</td>
<td>82</td>
<td>95</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

| Coinheritance frequencies^c for Em^Nov^ recombinants |     |     |     |     |     |     |     |     |     |     |
| Em    | 100 | 84  | 75  | 38  | 93  | 34  | 25  | 6   | 0   | 1   |
| Thr   | 84  | 100 | 88  | 48  | 79  | 44  | 37  | 20  | 16  | 17  |
| Trp   | 75  | 88  | 100 | 56  | 70  | 51  | 44  | 28  | 25  | 26  |
| Tyr   | 38  | 48  | 56  | 100 | 32  | 89  | 80  | 65  | 61  | 62  |
| Ala   | 93  | 79  | 70  | 32  | 100 | 40  | 30  | 13  | 7   | 7   |
| Tmn   | 34  | 44  | 51  | 89  | 40  | 100 | 89  | 69  | 66  | 66  |
| Ilv   | 25  | 37  | 44  | 80  | 30  | 89  | 100 | 78  | 75  | 75  |
| Ura   | 6   | 20  | 28  | 65  | 13  | 69  | 78  | 100 | 94  | 93  |
| Nov   | 0   | 16  | 25  | 61  | 7   | 66  | 75  | 94  | 100 | 99  |
| Mec   | 1   | 17  | 26  | 62  | 7   | 66  | 75  | 93  | 99  | 100 |

^aISP933 = Em^Thr^-Trp^-Tyr^-Ala^-Tmn^-Ilv^-Ura^-Nov^-Mec^.
ISP803 = Em^Thr^+Trp^+Tyr^+Ala^-Tmn^-Ilv^-Ura^-Nov^-Mec^.

^bNeither strain was irradiated and there was no selection during regeneration. The recombination frequency for the Em^Tmn^ selection was 3.4 x 10^6 per 1 x 10^9 cfu, and 616 recombinants were scored. The recombination frequency for the Em^Nov^ selection was 3.9 x 10^6 per 1 x 10^9 cfu, and 616 recombinants were scored.

^cThe numbers represent, for each pair of markers, the percentage of the total number of Em^Tmn^ or Em^Nov^ recombinants with either parent phenotype.
the TyrB marker. Interpretation of these results placed the orphan insertion to the left of thrB106 in linkage group I. High CIF values for both analyses were consistent with the pairs of markers known to be closely linked by transformation analysis. Moreover, the ilv-129 - uraA141 linkage was consistent for both selected classes of recombinants.

In previous fusions with ISP852 as the multiply-marked parent, the ala-126 marker scored easily and coinherited at high frequencies with markers that were known to be closely linked. However, when ISP933 was constructed (tyrB282::Tn551 ermB321 replaced lys-115 in ISP852) for use as the multiply-marked parent, Ala coinherited at low frequencies with Tmn and Ilv in linkage group III, markers known to be closely linked by transformation analysis.

The predicted position of Ω42[Chr::Tn551] was confirmed by transforming ISP926 with standard protocol DNA purified from ISP942 (Table 14). Of the Thr+ transformants, EmR was cotransformed 13% of the time and EmR segregated independently of Trp+. Among the EmR transformants, Thr+ cotransformed 6% of the time, while Ura- and Trp+ each cotransformed less than 1% of the time, respectively. As predicted by interpretations of fusion data, Ω42[Chr::Tn551] mapped between uraB232::Tn551 ermB327 and thrB106 within linkage group I.
Table 14. Transformation of ISP926 with standard protocol DNA purified from ISP942

<table>
<thead>
<tr>
<th>Relevant phenotypes</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
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<tr>
<td></td>
<td>Ura</td>
<td>Em</td>
<td>Thr</td>
<td>Trp</td>
</tr>
<tr>
<td>Donor:</td>
<td>-</td>
<td>r</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Recipient:</td>
<td>+</td>
<td>s</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Of 550 Thr⁺ transforms: 

- 68.9% (379)  
- 17.3% (95)   
- 12.7% (70)   
- 0.9% (5)     
- 0.2% (1) 

Of 1093 Em⁺ transforms: 

- 93.2% (1019)  
- 5.1% (56)     
- 0.9% (10)     
- 0.8% (8) 

\(^a\)The frequency of transformation and reversion was 305 per 1 x 10⁹ cfu and 50 per 1 x 10⁹ cfu, respectively.

\(^b\)The frequency of transformation and the rate of spontaneous resistance was 405 per 1 x 10⁹ cfu and 30 per 1 x 10⁹ cfu, respectively.

Fusion analysis of the purC193::Tn551 locus

The map location of purC193::Tn551, an orphan insertion mutation, was predicted from the CIF values in Table 15 to be linked to the thrB106 - trpE85 marker pair in linkage group I. As seen in the previous fusion analyses (Tables 11 and 13), high CIF values for both selection analyses were consistent with the pairs of markers known to be linked. In this experiment, crossovers were particularly rare between ThrB and TrpE as CIF
Table 15. Coinheritance frequencies for an ISP933 x ISP797\textsuperscript{a} protoplast fusion\textsuperscript{b}

<table>
<thead>
<tr>
<th></th>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
<th>Tyr</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
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<tbody>
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<td><strong>Em</strong></td>
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<td>92</td>
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<tr>
<td><strong>Nov</strong></td>
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<td>30</td>
<td>31</td>
<td>72</td>
<td>82</td>
<td>92</td>
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<tr>
<td><strong>Mec</strong></td>
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<td>30</td>
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<td>82</td>
<td>92</td>
<td>100</td>
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</tbody>
</table>

**Coinheritance frequencies\textsuperscript{c}** for Em\textsuperscript{Tmn} recombinants

<table>
<thead>
<tr>
<th></th>
<th>Em</th>
<th>Thr</th>
<th>Trp</th>
<th>Tyr</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
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<tbody>
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<tr>
<td><strong>Trp</strong></td>
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<td>96</td>
<td>100</td>
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<td>100</td>
<td>81</td>
<td>49</td>
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<td>43</td>
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<td><strong>Ilv</strong></td>
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<td><strong>Ura</strong></td>
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<td>43</td>
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<td><strong>Mec</strong></td>
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<td>62</td>
<td>94</td>
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</tbody>
</table>

\textsuperscript{a}ISP933 = Em\textsuperscript{S}Thr\textsuperscript{r}Trp\textsuperscript{r}Tyr\textsuperscript{r}Tmn\textsuperscript{r}Ilv\textsuperscript{r}Ura\textsuperscript{r}Nov\textsuperscript{r}Mec\textsuperscript{r}.

\textsuperscript{b}ISP797 = Em\textsuperscript{r}Thr\textsuperscript{r}Trp\textsuperscript{r}Tyr\textsuperscript{r}Tmn\textsuperscript{r}Ilv\textsuperscript{r}Ura\textsuperscript{r}Nov\textsuperscript{r}Mec\textsuperscript{r}.

\textsuperscript{b}Neither strain was irradiated and there was no selection during regeneration. The recombination frequency for the Em\textsuperscript{Tmn} selection was 1.8 \times 10^6 per 1 \times 10^9 cfu, and 616 recombinants were scored. The recombination frequency for the Em\textsuperscript{Nov} selection was 4.8 \times 10^6 per 1 \times 10^9 cfu, and 616 recombinants were scored.

\textsuperscript{c}The numbers represent, for each pair of markers, the percentage of the total number of Em\textsuperscript{Tmn} or Em\textsuperscript{Nov} recombinants with either parent phenotype.
values from the data of both selected classes were greater than 95%. The ala-126 marker was not included in the analysis since growth responses were difficult to distinguish.

The approximate location of purCl93::Tn551 was confirmed by transforming ISP997 with high molecular weight DNA from ISP797. Of 1094 Em^ transformants, 3.5% were Ura^ and none were Mec^ or Trp^-. The frequency of transformation and the rate of spontaneous resistance were 218 and 30 per \(1 \times 10^9\) cfu, respectively. Although the exact location of purCl93::Tn551 was not determined, the marker mapped to the left of thrB106 in the order purCl93::Tn551 - thrB - trp in which the relative order of uraB and purC was not determined.

**Fusion analysis of the 34[Chr::Tn551] locus** The following protoplast fusion experiment was designed with the thought of detecting close linkages among the end markers of the known linkage groups. The 34[Chr::Tn551] marker was chosen for use in this experiment because of its location on the "left" extremity of linkage group III, and its weak linkage (by transformation) to the tmn-3106 locus. ISP988 was used as the multiply-marked parent and was constructed through a series of transformations (refer to Table 1) which eliminated the ala-126 marker. The CIF values of both Nov^r Em^r and Tmn^r Em^r selections are presented in Table 16. A
Table 16. Coinheritance frequencies for an ISP988 x ISP796<sup>a</sup> protoplast fusion<sup>b</sup>

<table>
<thead>
<tr>
<th></th>
<th>Thr</th>
<th>Trp</th>
<th>Tyr</th>
<th>Em</th>
<th>Tmn</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coinheritance frequencies</strong>&lt;sup&gt;c&lt;/sup&gt; for Em&lt;sup&gt;+&lt;/sup&gt;Tmn&lt;sup&gt;+&lt;/sup&gt; recombinants</td>
<td></td>
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<td>60</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>Mec</td>
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<td>8</td>
<td>92</td>
<td>92</td>
<td>99</td>
<td>99</td>
<td>100</td>
</tr>
</tbody>
</table>

| **Coinheritance frequencies**<sup>c</sup> for Em<sup>+</sup>Nov<sup>+</sup> recombinants |     |     |     |    |     |     |     |     |     |
| Thr    | 100 | 90  | 41  | 41 | 47  | 54  | 60  | 59  | 59  |
| Trp    | 90  | 100 | 37  | 36 | 41  | 54  | 64  | 63  | 63  |
| Tyr    | 41  | 37  | 100 | 99 | 73  | 50  | 6   | 1   | 1   |
| Em     | 41  | 36  | 99  | 100| 72  | 49  | 4   | 0   | 0   |
| Tmn    | 47  | 41  | 73  | 72 | 100 | 76  | 32  | 28  | 28  |
| Ilv    | 54  | 54  | 50  | 49 | 76  | 100 | 55  | 51  | 51  |
| Ura    | 60  | 64  | 6   | 4  | 32  | 55  | 100 | 96  | 96  |
| Nov    | 59  | 63  | 1   | 0  | 28  | 51  | 96  | 100 | 100 |
| Mec    | 59  | 63  | 1   | 0  | 28  | 51  | 96  | 100 | 100 |

<sup>a</sup>ISP988 = Thr<sup>-</sup>Trp<sup>-</sup>Tyr<sup>-</sup>Em<sup>S</sup>Tmn<sup>+</sup>Ilv<sup>-</sup>Ura<sup>-</sup>Nov<sup>-</sup>Mec<sup>+</sup>

<sup>b</sup>ISP796 = Thr<sup+</sup>Trp<sup+</sup>Tyr<sup+</sup>Em<sup>-</sup>Tmn<sup>S</sup>Ilv<sup+</sup>Ura<sup+</sup>Nov<sup>S</sup>Mec<sup>+</sup>.

<sup>c</sup>Neither strain was irradiated and there was no selection during regeneration. The recombination frequency for the Em<sup>+</sup>Tmn<sup>+</sup> selection was 2.9 x 10<sup>6</sup> per 1 x 10<sup>9</sup> cfu, and 504 recombinants were scored. The recombination frequency for the Em<sup>+</sup>Nov<sup>+</sup> selection recombinants were scored.

<sup>c</sup>The numbers represent, for each pair of markers, the percentage of the total number of Em<sup>+</sup>Tmn<sup>+</sup> or Em<sup>+</sup>Nov<sup>+</sup> recombinants with either parent phenotype.
unique linkage was discovered as TyrB coinherited with Em at frequencies greater than 98%. Because tyrB282::Tn551 ermB321 occupied the extreme "right" end of linkage group I, these results were strongly indicative that linkage groups I and III were adjacent on the chromosome.

An attempt was made to confirm the linkage of tyrB282::Tn551 ermB321 and Ω34[Chr::Tn551] by transforming ISP991 with standard protocol DNA purified from ISP796. Among 1809 Em⁺ transformants, none were Tyr⁺. The frequency of transformation and the rate of spontaneous resistance were 1110 per $1 \times 10^9$ CFU and 50 per $1 \times 10^9$ CFU. After extensive modification of the standard DNA purification protocol, which included obtaining lysates from protoplasts and reducing or eliminating shear forces inherent in the experimental procedure, the experiment was repeated using high molecular weight DNA from ISP796 (Table 17). Among 2459 Em⁺ transformants, 2.5% were Tyr⁺; the Tyr and Rib phenotypes segregated independently, indicating that the order of markers was tyrB282::Tn551 ermB321 - Ω34[Chr::Tn551] - rib-127. The reciprocal selection confirmed this linkage; among 1228 Tyr⁺ transformants, 3.2% cotransformed with Em⁺. This same linkage was shown to be sensitive to DNA shearing treatments (Table 18). As the shear treatment of the DNA was progressively more harsh, cotransformation frequencies deteriorated at a faster rate than the Em⁺ transformation.
Table 17. Transformation of ISP991 with high molecular weight DNA from ISP796

<table>
<thead>
<tr>
<th>Relevant markers</th>
<th>Tyr</th>
<th>Em</th>
<th>Rib</th>
<th>Tmn</th>
</tr>
</thead>
<tbody>
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<td>Donor:</td>
<td>+</td>
<td>r</td>
<td>+</td>
<td>s</td>
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<tr>
<td>Recipient:</td>
<td>-</td>
<td>s</td>
<td>-</td>
<td>r</td>
</tr>
</tbody>
</table>

Of 2459 Em\(^r\) transformants \(^a\)

- 96.4% (2371)
- 2.5% (61)
- 0.6% (16)
- 0.4% (10)
- 0.1% (1)

Of 1228 Tyr\(^+\) transformants \(^b\)

- 96.8% (1189)
- 3.2% (39)

\(^a\) The frequency of transformation and rate of spontaneous resistance was 1250 per \(1 \times 10^9\) cfu and 50 per \(1 \times 10^9\) cfu, respectively.

\(^b\) The frequency of transformation and reversion rate per \(1 \times 10^8\) cfu and 10 per \(1 \times 10^9\) cfu, respectively.

\(^c\) Not scored.

Fusion analysis of the *tet-3490* locus  As a result of demonstrating that linkage groups I and III were linked by transformation, it only was necessary then to determine the proper orientation of linkage group II on a circular map.
Table 18. A test for the DNA shearing sensitivity of the
\textit{tyrB282::Tn551 ermB321 - 434[Chr::Tn551]} transformation linkage

<table>
<thead>
<tr>
<th>Shear treatment of DNA\textsuperscript{a}</th>
<th>(\text{Em}^r) transformation frequency\textsuperscript{b}</th>
<th>Frequency of cotransformation of (\text{Em}^r) and (\text{Tyr}^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\text{Number}</td>
<td>\text{Surviving fraction}</td>
</tr>
<tr>
<td>none</td>
<td>5190</td>
<td>1.00</td>
</tr>
<tr>
<td>16 ga.</td>
<td>4550</td>
<td>0.88</td>
</tr>
<tr>
<td>18 ga.</td>
<td>1630</td>
<td>0.31</td>
</tr>
<tr>
<td>21 ga.</td>
<td>840</td>
<td>0.16</td>
</tr>
<tr>
<td>no DNA</td>
<td>&lt;190</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Pooled ISP796 high molecular weight DNA (1.5 ml volumes) were rapidly forced once through a hypodermic needle attached to a 2-ml syringe; 0.15-ml portions were then added to 1-ml portions of competent cells containing \(1 \times 10^9\) cfu/ml.

\textsuperscript{b}\(\text{Em}^r\) transformants recovered per ml of transformation suspension.

\textsuperscript{c}Numbers in parentheses refer to the total numbers of \(\text{Em}^r\) transformants that were scored for the cotransformation of \(\text{Tyr}^+\).
of the chromosome. This question was approached by fusing ISP95 and ISP983. The former strain carries \textit{tet-3490}, a tetracycline-resistance determinant that was known not to be within the known linkage groups. ISP983, the multiply-marked parent, is a derivative of ISP933 in which \textit{ala-126} and \textit{tmn-3106} were displaced by moving in a silent \textit{Tn551} insertion, 05[Chr::Tn551]. The coinheritance frequency matrix for the \textit{Em^Tet^} class of recombinants (Table 19) shows high CIF values for Tet and the markers in linkage group II: Mec at 90\%, Nov at 88\%, and UraA at 84\%. Tet also coinherited at high frequencies with ThrB (72\%) and TrpE (71\%). Additional evidence for a \textit{mec-4916 - tet-3490 - thrB106} linkage is presented in the CIF matrix for the \textit{Nov^Tet^} class of recombinants (Table 19). Of the total \textit{Nov^Tet^} recombinants, 17\% had a crossover between Nov and Mec while only 2\% had a crossover between Nov and UraA. In previous fusion experiments, selection for \textit{Nov^} recombinants resulted in an average of 1 or 2\% of the total having crossovers in these 2 regions. The Tet - ThrB marker pair coinherited at a high frequency (41\%) as compared to other markers involved in the cross. The relatively low coinheritance of Tet with other markers can be explained by the high percentage (46\%) of recombinants in which the \textit{tet-3490} determinant was the only ISP95 marker inherited. This same phenotype was only 7.5\% of
Table 19. Coinheritance frequencies for an ISP983 x ISP95<sup>a</sup> protoplast fusion

<table>
<thead>
<tr>
<th></th>
<th>Thr</th>
<th>Trp</th>
<th>Tyr</th>
<th>Em</th>
<th>Ilv</th>
<th>Ura</th>
<th>Nov</th>
<th>Mec</th>
<th>Tet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coinheritance frequencies&lt;sup&gt;c&lt;/sup&gt; for Em&lt;sup&gt;+&lt;/sup&gt;Tet&lt;sup&gt;+&lt;/sup&gt; recombinants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>100</td>
<td>92</td>
<td>45</td>
<td>27</td>
<td>36</td>
<td>75</td>
<td>77</td>
<td>78</td>
<td>72</td>
</tr>
<tr>
<td>Trp</td>
<td>92</td>
<td>100</td>
<td>47</td>
<td>29</td>
<td>39</td>
<td>77</td>
<td>77</td>
<td>77</td>
<td>71</td>
</tr>
<tr>
<td>Tyr</td>
<td>45</td>
<td>47</td>
<td>100</td>
<td>72</td>
<td>68</td>
<td>39</td>
<td>38</td>
<td>37</td>
<td>28</td>
</tr>
<tr>
<td>Em</td>
<td>27</td>
<td>29</td>
<td>72</td>
<td>100</td>
<td>82</td>
<td>16</td>
<td>12</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ilv</td>
<td>36</td>
<td>39</td>
<td>68</td>
<td>82</td>
<td>100</td>
<td>34</td>
<td>30</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>Ura</td>
<td>75</td>
<td>77</td>
<td>39</td>
<td>16</td>
<td>34</td>
<td>100</td>
<td>96</td>
<td>95</td>
<td>84</td>
</tr>
<tr>
<td>Nov</td>
<td>77</td>
<td>77</td>
<td>38</td>
<td>12</td>
<td>30</td>
<td>96</td>
<td>100</td>
<td>98</td>
<td>88</td>
</tr>
<tr>
<td>Mec</td>
<td>78</td>
<td>77</td>
<td>37</td>
<td>10</td>
<td>29</td>
<td>95</td>
<td>98</td>
<td>100</td>
<td>90</td>
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<tr>
<td>Tet</td>
<td>72</td>
<td>71</td>
<td>28</td>
<td>0</td>
<td>18</td>
<td>84</td>
<td>88</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Coinheritance frequencies&lt;sup&gt;c&lt;/sup&gt; for Nov&lt;sup&gt;+&lt;/sup&gt;Tet&lt;sup&gt;+&lt;/sup&gt; recombinants</td>
<td></td>
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<td></td>
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<tr>
<td>Thr</td>
<td>100</td>
<td>93</td>
<td>68</td>
<td>59</td>
<td>59</td>
<td>60</td>
<td>59</td>
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<tr>
<td>Trp</td>
<td>93</td>
<td>100</td>
<td>73</td>
<td>63</td>
<td>63</td>
<td>64</td>
<td>63</td>
<td>72</td>
<td>37</td>
</tr>
<tr>
<td>Tyr</td>
<td>68</td>
<td>73</td>
<td>100</td>
<td>73</td>
<td>73</td>
<td>75</td>
<td>73</td>
<td>81</td>
<td>27</td>
</tr>
<tr>
<td>Em</td>
<td>59</td>
<td>63</td>
<td>73</td>
<td>100</td>
<td>99</td>
<td>98</td>
<td>99</td>
<td>82</td>
<td>1</td>
</tr>
<tr>
<td>Ilv</td>
<td>59</td>
<td>63</td>
<td>73</td>
<td>99</td>
<td>100</td>
<td>99</td>
<td>99</td>
<td>83</td>
<td>1</td>
</tr>
<tr>
<td>Ura</td>
<td>60</td>
<td>64</td>
<td>75</td>
<td>98</td>
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<td>98</td>
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<td>Nov</td>
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<td>73</td>
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<td>98</td>
<td>100</td>
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</tr>
<tr>
<td>Mec</td>
<td>69</td>
<td>72</td>
<td>81</td>
<td>82</td>
<td>83</td>
<td>81</td>
<td>83</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Tet</td>
<td>41</td>
<td>37</td>
<td>27</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>17</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> ISP983 = Thr<sup>−</sup>Trp<sup>−</sup>Tyr<sup>−</sup>Em<sup>−</sup>Ilv<sup>−</sup>Ura<sup>−</sup>Nov<sup>−</sup>Mec<sup>−</sup>Tet<sup>+</sup>

ISP95 = Thr<sup>+</sup>Trp<sup>+</sup>Tyr<sup>+</sup>Em<sup>+</sup>Ilv<sup>+</sup>Ura<sup>+</sup>Nov<sup>+</sup>Mec<sup>+</sup>Tet<sup>+</sup>.

<sup>b</sup> Neither strain was irradiated and there was no selection during regeneration. The recombination frequency for the Em<sup>+</sup>Tet<sup>+</sup> selection was 4.5 x 10<sup>5</sup> per 1 x 10<sup>9</sup> cfu, and 560 recombinants were scored. The recombination frequency for the Nov<sup>+</sup>Tet<sup>+</sup> selection was 1.1 x 10<sup>5</sup> per 1 x 10<sup>9</sup> cfu, and 448 recombinants were scored.

<sup>c</sup> The numbers represent, for each pair of markers, the percentage of the total number of Em<sup>+</sup>Tet<sup>+</sup> or Nov<sup>+</sup>Tet<sup>+</sup> recombinants with either parent phenotype.
the total in the analysis for the Em$^r$Tet$^r$ recombinants.

**Summary of fusion and transformation results**

Interpretations of fusion and transformation analyses with high molecular weight DNA have resulted in the construction of the chromosome map as presented in Figure 2. The orphan Tn$^{551}$ insertions have all mapped to the "left" of ThrB in segments 12 and 13. TyrB and R34 were shown to be closely linked by fusion and the linkage was confirmed by transformation (segment 2). The mec-4916 - tet-3490 - thrB106 markers were linked in that order by fusion but only a very weak linkage of Mec and Tet was seen by transformation (data not shown). Finally, fusion data from several experiments have suggested the remaining linkage between uraAl41 and ilv-129.
Figure 2. The *S. aureus* chromosome map. All of the markers connected by solid lines have been linked by transformation analysis. The dashed lines connect markers that have been linked by protoplast fusion results.
DISCUSSION

Experimental Conditions for Protoplast Fusion

When compared to generalized transduction (Morse, 1959) and transformation (Lindberg et al., 1972), relatively high frequencies of recombination were observed in S. aureus protoplast fusions. In 9- and 10-factor genetic crosses, the frequencies varied from $1 \times 10^{-5}$ to $1 \times 10^{-2}$ among selected recombinants, and of the total viable regenerated cells, approximately 6% were recombinant phenotypes. Because of the high frequencies of recombination, the performance of certain types of experiments other than chromosome mapping was made possible. The segregation of genetic markers among recombinant phenotypes could be studied by fusion analysis without introducing selection in the experiment. Also, theoretically any genetic traits, even those which cannot normally be selected, could be combined in the same genetic background.

Probably the most effective use of the types of experiments described above would require a study of the optimum conditions for protoplast fusion to obtain the highest possible recombination frequencies. An alternative approach would be to enrich for recombinant phenotypes by exposing the parents to UV radiation, but undesirable side effects have been
The primary objective of this study was to develop fusion analysis as a supplementary technique for chromosome mapping in *S. aureus*, and to learn more about the arrangement of the established linkage groups on a circular map. Although high recombination frequencies were desirable and perhaps to a certain extent necessary, they were secondary to developing experimental conditions for fusion analysis that simulated a random exchange of genetic information.

Some of the early experiments that determined the conditions necessary for the recovery of high proportions of recombinant phenotypes were interpreted cautiously. As the data in Table 10 show, the frequencies of recombination of repeated experiments varied by as much as ±50%. It was encouraging to discover, however, that the distribution of unselected markers in the crosses was reproducible, at least within about 5%.

Protoplast fusion can be subdivided into three successive experimental steps: 1) the production of protoplasts, 2) protoplast fusion, and 3) the regeneration of fused protoplasts. The most important considerations for the production of protoplasts are the efficiency of protoplast formation and the percentage recovery of viable protoplasts after treatment with the enzyme. These requirements were met...
successfully in *S. aureus* fusions as less than 1 cell in $1 \times 10^9$ protoplasts formed a colony on BHI agar without sucrose; in addition, 22% of the total cells exposed to the enzyme survived as protoplasts. The high efficiency of protoplast formation can be attributed, in part, to the mixing of the cell-protoplast suspension on a roller-extractor apparatus. When reciprocal shaking was used to agitate this suspension, the incidence of nonprotoplasted units was significantly higher.

Low molecular weight PEG solutions (less than 1000) have been reported to be toxic to plant protoplasts (Kao and Michaylak, 1974; Kao et al., 1974). Because of this, it seems, low molecular weight PEG has been avoided for use with protoplast fusions of bacteria. There are several obvious advantages in using the low molecular weight PEG solutions: 1) solutions of PEG 200 or 400 at concentrations of 10 to 100% (v/v) are fluid at ice bath temperatures, 2) because of the increased fluidity, suspensions of protoplasts in PEG are more easily mixed and handled, and 3) because high PEG concentrations can be manipulated, the effects of DMSO, a fusion enhancer, can be easily determined.

PEG 1000 is a solid at room temperature and solutions containing more than 60% (v/v) solidify at ice bath temperatures (8-10 C). Because concentrations of PEG 1000
containing more than 60% could not be used for fusion, the highest recombination frequencies were observed when DMSO was included in the 60% PEG solution (Table 6). In fusions with both PEG 200 and 400 (Tables 7 and 8), the enhancing effect of DMSO could be overcome by increasing the concentration of PEG in the solution.

Senda et al. (1980) speculated that the fusion process was, in part, regulated by membrane fluidity and that membrane fluidity was a function of the temperature at which the fusion mixtures were held. This suggested that the efficiency of protoplast fusion with varying concentrations of PEG was indirectly related to the temperature at which the protoplasts were fused. Within certain limits, fusions with predesignated concentrations of PEG (40 to 70% (v/v)) might be optimized simply by varying the temperature at which the protoplasts are held.

A small number of protoplasts gave rise to more rapid growth into colonies on R medium. These early-forming colonies were surrounded by a zone within which the regeneration of other protoplasts was inhibited. This so-called "crowding out" effect has been observed by others (Landman and DeCastro-Costa, 1976; Decastro-Costa and Landman, 1977; Hopwood et al., 1977); it has been suggested that in B. subtilis this zone reflects the production of an autolysin
by cells within the early-forming colony (Landman and DeCastro-Costa, 1976; DeCastro-Costa and Landman, 1977).

DeCastro-Costa and Landman (1977) reported that high agar concentrations (2-3%) in regeneration medium reduced the diffusion of this regeneration-inhibition-factor and restricted the development of large colonies. When regenerating fused protoplasts of *S. aureus* on R medium, an approximate ten-fold increase in regeneration frequencies was observed when 2.5% agar was substituted for 1.5% agar; crowding-out effects also were reduced significantly.

Akamatsu and Sekiguchi (1981) found that polyvinyl pyrrolidone or dextran could replace horse serum, bovine serum or gelatin as stimulators of regeneration for *B. subtilis* protoplasts. Starch (0.3%), when added to R medium, increased the regeneration of *S. aureus* protoplasts by a factor of 10; perhaps starch has a similar mechanism to that of dextran or polyvinyl pyrrolidone in stimulating protoplast regeneration.

Humidity control during regeneration seemed to be a minor consideration with other fusions systems. With some *Streptomyces* spp. (Hopwood, 1981), a considerable dehydration of the base agar layer (20% water loss) stimulated regeneration of protoplasts in the upper soft agar layer. In *S. aureus* fusions, humidity control during regeneration was an important consideration for obtaining consistent results. The plates
were predried at 35 C for 12-24 h, or until the surface moisture had dried; immediately after inoculation, these plates were placed in a humidified incubator for the duration of incubation. If the plates dried too quickly during incubation or if they were predried insufficiently, recombination and regeneration frequencies were reduced.

**Genetic Analysis by Protoplast Fusion**

There was a significant amount of evidence presented in these studies showing that the observed recombination frequencies were a result of protoplast fusion. Both protoplast formation and PEG were required to obtain recombination and the presence of DNase had no effect. The observed recombination frequencies were several magnitudes higher than those observed by transformation (Lindberg et al., 1972) or generalized transduction (Morse, 1959). Moreover, markers that were completely unlinked as measured by transformation or transduction were coinherited at high frequencies during protoplast fusion.

**Experimental conditions necessary for chromosome mapping**

The following modifications were made in the fusion analysis procedure that simulated a random bidirectional exchange of marker alleles between parents and allowed a
random sampling of recombinants for analysis: 1) The ratio of the number of protoplasts of each parent fused was approximately 1 to 1. 2) No selection was made during regeneration and growth on R medium. 3) The cells that were recovered from 5 regeneration plates were pooled, sonicated to disperse cell aggregates, and plated onto selective media. 4) Isolated clones were picked (400-600 per selection) from the selection plates at random and were analyzed for the distribution of unselected markers.

The recombinants obtained by protoplast fusion were assumed to be stable haploids. In support of this assumption were the facts that the phenotypes of fusion recombinants were stable upon repeated subculturing and acted as true haploids when used as recipients or as a source of donor DNA in transformations. Also, the biparentals or diploids that were isolated from B. subtilis protoplast fusions by Hotchkiss and Gabor (1980) were noncomplementary; consequently, the diploids did not grow on media selective for recombinant phenotypes.

Even under the experimental conditions described above, growth was probably more favorable for those recombinants that inherited the least number of markers that conferred nutritional deficiencies. This conclusion was based partially on the fact that the multiply-marked parent grew at
approximately one-half the rate of the prototrophic parent. The results of an ISP933 x ISP808 fusion confirmed this as 1200 clones (isolated under nonselective conditions) were analyzed for the distribution of marker alleles. Seventy-five percent of the nonrecombinant clones were ISP808 phenotypic (prototrophic parent) and only 25% were ISP933 phenotype (multiply-marked parent). An alternative explanation for these results would be that the multiple-marked parent was less likely to survive the protoplasting treatment. If the yields in protoplasts were significantly lower for one parent, then deviations from the 1 to 1 fusion ratio would be expected.

Fodor and Alfoldi (1979), in fusions with B. megaterium, did not obtain consistent linkage data. They observed significant variations in the distribution of markers among the recombinants simply by changing the composition of the medium in which the parent strains were grown before protoplasting. Their procedure, when compared to S. aureus fusion analysis, differed in that cells for protoplast formation were grown in minimal media and recombinants were selected directly during regeneration.

Schaeffer et al. (1976) in working with B. subtilis and Hopwood and Wright (1978), using S. coelicolor, grew the parent strains for protoplast formation in complex media and
regeneration was nonselective. Both groups found that linkage relationships were consistent with the known linkage maps. Baltz (1980) proposed a rudimentary linkage map in *Streptomyces fradiae* on the basis of fusion analysis by arranging the markers involved in the cross so that the incidence of multiple crossovers was minimal.

Hopwood and Wright (1981), in fusions with *S. coelicolor*, found that when one or both parents were irradiated with UV light, the incidence of multiple crossovers increased, and in some cases, known linkage relationships deteriorated. When the conditions for simulating a unidirectional exchange of genetic information were incorporated into *S. aureus* fusion analysis (UV inactivation of the donor parent and selection against the survival of the donor during regeneration), the approximate location of Ω1028[Chr::Tn551] was predicted. Although, in this case, the modified procedure was successful, the changes were eliminated because of the possible deleterious effects of UV radiation on linkage relationships.

**Genetic markers and parent strains**

The most difficult and time-consuming aspect in developing fusion analysis for chromosome mapping in *S. aureus* was the construction of multiply-marked parents with useful markers in strategic map locations. In many early fusion
crosses, colonies growing on selective media were found to be irregular in shape and small in diameter. It appeared that cells within the colony had been infected by exogenous bacteriophage. These "mottled" colonies were not observed in later fusions when only parents that exhibited the same phage-typing pattern were crossed.

Many phenotypes were expressed only rarely among fusion recombinants and as a result, were not coinherited at high frequencies with other markers known to be closely linked. These included Pig\(^+\), Thy\(^-\), His\(^-\), PurA\(^-\), and, in some genetic backgrounds, Ala\(^-\). Because of phenomena such as these, strategies in the construction and selection of markers used in fusion analysis were considered carefully. Antibiotic resistance determinants were found to be an excellent choice for use in fusion analysis; the resistance markers used in this study coinherited predictably with markers known to be linked, and in most cases (other than with some Tn551 insertion mutations) did not confer added nutritional deficiencies.

Interpretation and analysis of fusion data

The use of a programmed minicomputer to sort the scoring data into phenotypes and the frequencies at which they occurred was essential in developing chromosome mapping by protoplast fusion. The scoring data for each experiment were
stored for future use on a magnetic disk. Thus, new methods for the treatment of fusion data were tested on a large backlog of data stored from previous experiments.

Several attempts were made to obtain quantitative linkage data (map distances) with fusion analysis, but coinheritance frequencies of linked markers varied significantly depending upon the map locations of the selected markers.

A consistent and unexplained anomaly was the high frequency of crossovers between tyrB282::Tn551 ermB321 and trpE85. These markers were known to be linked by transformation, but high coinheritance frequencies by fusion were rare. Gabor and Hotchkiss (1981), in B. subtilis fusions, found that crossover activity near the origin and terminus of the chromosome was 20 times more frequent when compared to other regions of the chromosome.

Transposable elements have been shown to induce deletions, inversions, and tandem duplications within replicons (Kleckner et al., 1977). Consequently, linkage data that involve possible interactions between Tn551 elements inserted at different loci must be interpreted cautiously.

Interactions (homologous recombination) between Tn551 elements during protoplast fusion may have contributed to high CIF values (98%) observed for the Ω34 - TyrB marker pair (Table 16). Two possible interactions between these
Tn551 insertions can be envisioned: 1) the ermB determinant in Ω34[Chr::Tn551] repaired the ermB321 mutation in tyrB282::Tn551 by homologous recombination, and 2) homologous recombination between insertions resulted in a chromosomal deletion of the DNA between the 2 loci. The former interaction could not have contributed to a high CIF value since the resulting phenotype would have been Em^Tyr^-; this is a recombinant phenotype and actually would have decreased the Ω34 - TyrB CIF value. A deletion between the Tn551 elements would have resulted in 2 possible phenotypes, Tyr^-Em^S and Tyr^-Em^R, and was unlikely for at least 2 reasons. The Em^S phenotype was selected against and a chromosomal deletion of that magnitude probably would have been lethal.

The most convincing evidence for an Ω34[Chr::Tn551] - tyrB282::Tn551 ermB321 linkage was confirmation by reciprocal transformations with high molecular weight DNA. Any interactions between Tn551 insertions during transformation (similar to those discussed above) should have been prevalent regardless of the molecular weight of the DNA used. However, linkage was not demonstrated with sheared high molecular weight DNA (Table 18) or with DNA purified according to the standard protocol (low molecular weight DNA).
A summary of protoplast fusion as a chromosome mapping technique

The following is a summary of the contributions that were made to simplify chromosome mapping in *S. aureus*: 1) A protoplast fusion procedure was developed that simulated bidirectional and random genetic exchange between parents in the cross. 2) A computer-assisted analysis procedure was developed with which data was entered directly into the computer from replica plates; and coinheritance frequencies for all possible pairs of markers were generated in the form of a 2-dimensional matrix. Interpretations of these data allowed the user to predict the relative map order of markers involved in the cross. 3) Through the use of fusion analysis, the relative map positions of 4 orphan markers were predicted and the positions of 3 were confirmed by transformation analysis. 4) The relationships of markers known to be linked were entirely consistent with data accumulated by fusion analysis. 5) Fusion data were accumulated that positioned the 3 linkage groups on a circular map; one of the linkages, "tyrB282::Tn551 ermB321 - Ω34[Chr::Tn551]", was confirmed by transformation analysis. 6) A procedure was developed for isolating high molecular weight transforming DNA from protoplasts. This DNA was instrumental in confirming, by transformation, some of the linkages predicted by fusion analysis.

There are 2 possible approaches for mapping new markers
in *S. aureus* 8325: 1) predict the relative map location of the marker by fusion analysis and confirm it by transformation with high molecular weight DNA, or 2) use transformation alone. Transformation analysis with high molecular weight DNA would be highly effective for mapping new markers if the marker mapped either within or sufficiently near the markers of the known linkage areas. However, there are 2 chromosomal gaps of unknown dimension yet to be characterized by transformation; the *uraA*141- *pig*131 region and the *mec*4916 - *tet*3490 - *uraB*232 region. Fusion analysis would determine the probable location of orphan markers within the gaps; transformation analysis alone would not. This information would allow the construction of additional linkage groups, by transformation, within the gaps and eventually a circular map could be defined by quantitative transformation data.
LITERATURE CITED


ACKNOWLEDGMENTS

I would like to extend my appreciation to Dr. Peter A. Pattee for his guidance and professional advice and for those frequent early-morning "talks about science", which were a valuable part of my graduate education. Finally, and most sincerely, I thank my wife Susan for her love and friendship throughout my graduate career.

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APPENDIX: FUSION ANALYSIS PROGRAMS
READY.

100 PRINT"";
103 POKE3468.12
105 POKE 144.88:REM INACTIVATES R/S KEY
110 PRINT"THIS IS A PROGRAM FOR PROTOPLAST"
115 PRINT"FUSION DATA ANALYSIS"
130 FORX=1TO1000:NEXT
140 PRINT"THIS IS PART OF THE PROGRAM":FORX=1TO1000:NEXT
150 PRINT"DO YOU WANT PROGRAM INSTRUCTIONS? (Y/N)"
160 GETA$:IFA$=""THEN160
170 IF$=CHR$(13)THEN160
180 IF$="Y"THENGOSUB2700:GOTO220
190 IF$="N"THENGOTO220
200 GOTO160
210 GOTO62999
220 PRINT""
230 PRINT "YOU WISH TO SORT FOR ANALYSIS"
237 PRINT "DO NOT INCLUDE CONTAMINANTS OR MISSING SLOTS IN THIS TOTAL"
238 PRINT
240 INPUTA
244 PRINT
245 PRINT"ENTER THE TOTAL # OF RECOMBINANTS YOU WISH TO SORT FOR ANALYSIS"
247 PRINT
250 INPUTS
260 PRINT"ENTER THE # OF UNSELECTED MARKERS IN THIS CROSS"
264 PRINT
270 PRINT:PRINT" YOU HAVE MADE ROOM FOR"
280 PRINT"IS THIS CORRECT? (Y/N)"
290 GETC$:IFC$=""THEN290
300 IF$=CHR$(13)THEN290
310 IF$="Y"THEN440
320 IF$="N"THENCLR:PRINT"":GOTO230
330 GOTO390
340 GETC$:IFC$=""THEN340
350 GOSUB920:GOTO760
360 PRINT""
370 PRINT"CONSULT THE PPF LOGBOOK AND ASSIGN"
380 PRINT
390 GETC$:IFC$=""THEN390
400 IF$=CHR$(13)THEN390
410 IF$="Y"THEN440
420 IF$="N"THENCLR:PRINT"":GOTO230
430 GOTO390
440 GOSUB920:GOTO760
445 REM THIS SUBROUTINE WRITES XX%, PKT$, PKT, R, B, PK, AND MM TO
447 REM THE DISK IN A SEQUENTIAL FILE.
450 PRINT""
455 PRINT"CONSULT THE PPF LOGBOOK AND ASSIGN"
460 PRINT""
470 PRINT"INSERT DISK IN DRIVE1"
480 PRINT"TYPE IN A FILENAME":PRINT
490 FD$=""
500 INPUTFD#
510 PRINT"PRESS <RETURN> WHEN DISK IS READY TO WRITE"
520 GETD$:IFD$=""THEN520
530 OPEN15,8.15
540 GOSUB730
550 OPEN1,8,4,"1:"+F$+,"SEQ,WRITE"
560 PRINT"WRITING...."
570 PRINT#1,A;CHR$(13);B;CHR$(13);PK;CHR$(13);
580 CLOSE1
590 FL$=F$+"a"
600 OPEN3,8,4,"1:"
FL$+,"SEQ,WRITE"
610 CLOSE2
620 FORX=1TOB
630 PRINT#3,M$<X>;CHR$(13);
640 NEXTX
650 FORQQ=1TOPK
660 PRINT#3,PK$<QQ>;CHR$(13);
670 NEXTQQ
680 FORRR=1TOPK
690 PRINT#3,PK$(RR);CHR$(13);
700 NEXTRR
710 CLOSE3;CLOSE15
720 RETURN
725 REM *********** DOS ERROR SUBROUTINE ************
730 INPUT#15,A$,B$,C$,D$
740 IF VAL$m$<:0 THEN PRINTA$, B$, C$.. D$ : CLOSE3 : CLOSE15 : STOP
750 RETURN
760 PRINT"3"
770 PRINT"*M" ; : FORX=1TOB : PRINT"X" ; MM$(X) ; NEXT ; PRINT"Mi®!l"
780 PRINT"IS THIS CORRECT? <Y/N)"
790 GETC$; IF C$="" THEN 790
800 IF C$=CHR$(13) THEN 790
810 IF C$="N" THEN GOSUB 920: GOTO 760
820 IF C$="Y" THEN 1180
830 REM**********************************************************************
831 REM SUBROUTINE FOR SORTING THE PHENOTYPES (LOADS PK$< ) AND PK$< ).
832 REM**********************************************************************
840 IF CT=1 THEN PK=1:PK$=PT$:CT=1:GOTO 890
850 F=0:FORPX=1 TO PK
860 IF PK$<PX>=PT$ THEN PT$<PX>=PT$<PX>+1:F=1
870 NEXT
880 IF F=0 THEN PK=PK+1:PK$=PT$:CT=CT+1:GOTO 890
890 PT$=""
900 RETURN
920 REM "******** LOADING THE MM$< ) ARRAY WITH MARKER DESIG. *********
925 REM
930 PRINT"THERE ARE 2 MARKERS IF I RECALL."
940 INPUT"PLEASE INPUT A THREE LETTER ABBREVIATION":MM$(X);""
950 NEXTX
960 RETURN
970 REM********************************************************************
980 REM REVERSES PARTS OF THE MM$< ) MATRIX DEPENDING UPON THE WAY A MARKER
990 REM WAS SCORED ( MD$< ) (M/G) OR WHETHER THE MARKER WAS FROM THE DONOR
1000 REM OR THE RECIPIENT ( MR$< ) (D/R)).
1010 REM********************************************************************
1020 PRINT"^" : PRINT")!MGRAB A BEER, SIT BACK, AND RELAX ! ! !"
1030 PRINT")!WORKING...."
1040 FORXX=1TOB
1050 IFMR$<XX>="R" AND MD$<XX>="M" THEN 1120
1100 REM*********************** DOS ERROR SUBROUTINE ***************************
1120 PRINT"3"
1100 IF MS$(XX) = "S" AND MD$(XX) = "O" THEN 1120
1110 GOTO 1150
1120 FOR V = 1 TO CT
1130 IF X%(V, XX) = 0 THEN X%(Y, XX) = 1: GOTO 1150
1140 IF X%(Y, XX) = 1 THEN X%(Y, XX) = 0
1150 NEXT
1160 NEXT
1170 RETURN
1175 REM**********SUBRTS. FOR LOADING THE MS$(XX) AND MD$(XX) ARRAYS **********
1180 GOSUB 1960: GOSUB 2270
1185 REM ******************************************************************
1186 REM THIS AREA LOADS THE XX/XX MATRIX BY COLUMN (BY MARKER NOT BY PHENOTYPE
1187 REM********************************************************************
1190 PRINT "J"
1200 PRINT "J5KPRESS ïPË FOR POSITIVE" : PRINT "W«PRESS SPACE BAR" FOR NEGATIVE"
1210 PRINT \";«PRESS dll FOR A CONTAMINANT OR A" ••PRINT "MISSING COLONY"
1220 PRINT \"%D!PRESS SBHIFT QB FOR AN OPTION MENU"
1230 FOR X = 1 TO 1000 : NEXT
1240 INPUT \"]mmMOW MANY MASTER PLATES DO YOU HflVE"; Q?
1250 FOR X = 1 TO B: PRINT \"3%PLEASE SCORE PLATES 1 -"; Q; ";OF THEMS"
1260 PRINT \" S"; MMS < X >; •" ;"MARKER": FOR P = 1 TO 2000: NEXT
1265 PR I NT "T"
1270 R = 0
1272 REM************************************************************************
1273 REM 0% = # OF MASTER PLATES
1274 REM S% = # OF CONTAMINANTS OR MISSING COLONIES ON THE PLATE BEING SCORED.
1275 REM U% = # OF RECOMBINANTS ON THE PLATE BEING SCORED.
1276 REM D% = # OF RECOMBINANTS ON THE PREVIOUS PLATE.
1277 REM************************************************************************
1280 FOR Y = 1 TO Q%: S% = 0: U% = 0
1281 PRINT \"PRESS FOR POSITIVE";
1282 PRINT \"PRESS SPACE BAR FOR NEGATIVE"
1283 PRINT \"PRESS ïFor A CONTAMINANT OR A"
1284 PRINT \"MISSING COLONY"
1285 PRINT \"SHIFT Q! FOR AN OPTION MENU"
1290 PRINT \" J"
1295 PRINT \"J""; MMS<Y>; •"; "J"
1300 REM********** MASTER PLATE SCREEN DISPLAY ****************************
1310 FOR Q = 49 TO 57 READ DE.. FD = POKE DE.. QP : POKEFD, 32 : NEXT
1320 FOR Q = 49 TO 53
1330 FOR Q = 48 TO 57
1340 READ AB, BC: POKE AB, QP: POKE BC, SG
1350 IF Q = 53 AND Q = 54 THEN G = 57
1360 NEXT: RESTORE
1370 PRINT \"J"
1380 R = R + 1: U% = U% + 1
1390 GOSUB IF• = "THEN1390"
1400 IF• = "THENMSUB1460:XX(R.X) = 1: GOTO 1500
1410 IF• = "THENMDSUB1460:XX(R.X) = 0: GOTO 1510
1420 IF• = "THEN1480"
1430 IF• = "CHR$(269):THEN2550"
1440 IF• = "CHR$(13):GOTO1450"
1450 GOSUB 1780: R = R + 1: U% = 0: S% = 0: RESTORE: GOTO 1281
1460 IF• = "ATHENOSUB1780:RESTORE:GOTO 1270"
1470 IF• = "THENRETUrn"
1480 IF• = "56THENREADAB.BC:POKEAB.66:POKEBC.66:R=R-1:GOTO 1550"
1490 READAB.BC:POKEAB.66:POKEBC.66:R=S = S + 1: GOTO 1590
1500 READAB.BC:POKEAB.102:POKEBC.102:GOTO 1520
1510 READAB.BC:POKEAB.87:POKEBC.87
1520 IF• = "S"<56THEN1380
1530 IFUX+S%=56THEN1550
1540 IFR=ATHEN1550
1550 PRINT"DO YOU? (Y/N)"
1560 GETA$:IFm$=""THEN1560
1570 IFm$="V"THENRESTORE:GOTO1610
1580 IFm$="N"THENPRINT"Y":R=R-U%:U%=0:S%=0:RESTORE:GOTO1290
1590 IFm$=CHR$(209)THEN2550
1600 GOTO1660
1610 PRINT"Y"
1620 U%=U%
1630 NEXT
1640 CT=R
1650 NEXT:GOSUB1660
1655 REM********* CONTROL SUBRT FOR SORTING PHENOTYPES **************************
1660 PRINT:PRINT"SORTING PHENOTYPES..."
1670 FORC=1TOA
1680 FORX=1TOB
1690 IFX%(CT,XX)=0THENA$="D"
1700 IFX%(CT,XX)=1THENA$="R"
1710 PT$=PT$+m$
1720 NEXT
1730 GOSUB3840
1740 NEXT
1750 CT=A
1755 REM*********** POKE STATEMENT REACTIVATES R/S KEY *************************
1760 GOSUB450:POKE144,85:REM ACTIVATES R/S KEY
1765 CLR
1770 LOAD":\FUSION PART2",8
1780 PRINT"Y"
1790 PRINT"YOU HAVE MADE AN ERROR"
1800 PRINT"PLEASE RESCORE THIS PLATE"
1810 PRINT"PRESS <RETURN> TO CONTINUE"
1820 GETS$:IFS$=""THEN1820
1830 IFS$=CHR$(13)THEN1850
1840 GOTO1920
1859 PRINT"Y"
1860 RETURN
1865 REM********************************************************************
1870 REM AN ERROR SUBRT FOR DATA TALLYING WHEN THE OPERATOR MAKES A MISTAKE IN
1875 PRINT"YOU MUST DESIGNATE EACH MARKER AS BEING
1877 REM ACCOUNTING FOR ALL THE CONTAMINANTS OR MISSING COLONIES.
1879 REM********************************************************************
1888 PRINT"YOU HAVE MADE A COSTLY SUBSCRIPT ERROR"
1890 PRINT"START WITH MASTER#1 OF THIS MARKER":PRINT"AND TRY AGAIN"
1898 PRINT"YOU HAVE ACCOUNTED FOR ALL THE CONTAMINANTS?"
1910 PRINT"PRESS <RETURN> TO CONTINUE"
1920 GETA$:IFS$=""THEN1920
1930 IFS$=CHR$(13)THEN1950
1940 GOTO1950
1950 PRINT"Y":RETURN
1955 REM********************************************************************
1956 REM DISTINGUISHES EACH MARKER AS BEING FROM THE DONOR (D) OR RECIPIENT
1957 REM (R) STRAIN. LOADS THE MR$() ARRAY.
1959 REM********************************************************************
1960 PRINT"Y"
1970 PRINT"YOU MUST DESIGNATE EACH MARKER AS BEING"
1980 PRINT"PRESS D FOR THE DONOR OR PRESS R FOR RECIPIENT"
1990 PRINT"PRESS D FOR STRAIN":PRINT"ADD"
2000 FORX=1TOB:PRINT"":PRINTMR$(X);:NEXT
100

1010 AE=33810
1020 FORX=1TOB:A$=""'
1020 GETAS:IFAS="THEN2350
1030 IFAS="D"THENM$(X)=M$(X)+A$:POKERE,E,132:GOTO2080
1030 IFAS="R"THENM$(X)=M$(X)+A$:POKERE,E,146:GOTO2080
1030 IFAS=CHR$(13)THEN2350
1070 GOTO2080
1080 AE=AE+4:NEXT
1090 PRINT:PRINT:PRINT:PRINT"OK? (Y/N)"
1090 GETB$:IFB$=""THEN1960
1110 IFB$="Y"THEN1960
1110 IFB$="N"THEN1960
1130 IFB$=CHR$(13)THEN1960
1140 GOTO2080
1150 RETURN
1150 REM*********** DATA SET FOR THE POKE TO SCREEN CMDS ***************
1150 DATA 32862,32863,32867,32868,32871,32872,32873,32893,32894,32895,32896
1150 DATA 32897,32900,32901,32902,32903,32904,32905,32906,32907,32908,32909
1150 DATA 32910,32911,32912,32913,32914,32915,32916,32917,32918,32919,32920
1150 DATA 32921,32922,32923,32924,32925,32926,32927,32928,32929,32930,32931
1150 DATA 32932,32933,32934,32935,32936,32937,32938,32939,32940,32941,32942
1150 DATA 32943,32944,32945,32946,32947,32948,32949,32950,32951,32952,32953
1150 DATA 32954,32955,32956,32957,32958,32959,32960,32961,32962,32963,32964
1150 DATA 32965,32966,32967,32968,32969,32970,32971,32972,32973,32974,32975
1150 DATA 32976,32977,32978,32979,32980,32981,32982,32983,32984,32985,32986
1150 DATA 32987,32988,32989,32990,32991,32992,32993,32994,32995,32996,32997
1150 DATA 32998,32999,33000,33001,33002,33003,33004,33005,33006,33007,33008
1150 DATA 33009,33010,33011,33012,33013,33014
1150 REM*****#******#*****************************W*************************
1150 REM LOADS THE MD$(X) ARRAY WHICH DISTINGUISHES THE MARKERS AS TO HOW THEY
1150 REM ARE SCORED. GROWTH (G) OR NO GROWTH (N).
1150 REM********************************************************************
1160 PRINT"3"'
1160 PRINT"YOU MUST ALSO DESIGNATE THE NATURE OF"
1160 PRINT"THE SCORED MARKER. PRESS # FOR GROWTH"
1160 PRINT"OR PRESS #! FOR NO GROWTH (E.G. AUXOTROPHIC DET.)"
1160 PRINT"FOR NO GROWTH (E.G. AUXOTROPHIC DET.)":PRINT""'
1160 FORX=1TOB:PRINT".":PRINTM$(X):=NEXT
1160 AE=33810
1160 AE=AE+4:NEXT
1170 PRINT:PRINT:PRINT:PRINT"OK? (Y/N)"
1170 GETB$:IFB$=""THEN1960
1190 IFB$="Y"THEN1960
1190 IFB$="N"THEN1960
1210 IFB$=CHR$(13)THEN1960
1220 GOTO2080
1230 RETURN
1230 RETURN
1230 REM*********** SALVAGE MENU FOR ERRORS IN TALLYING DATA *************
1230 PRINT"3"'
1230 PRINT"YOU MUST ALSO DESIGNATE THE NATURE OF"
1230 PRINT"THE SCORED MARKER. PRESS # FOR GROWTH"
1230 PRINT"OR PRESS #! FOR NO GROWTH (E.G. AUXOTROPHIC DET.)"
1230 PRINT"FOR NO GROWTH (E.G. AUXOTROPHIC DET.)":PRINT""'
1230 FORX=1TOB:PRINT".":PRINTM$(X):=NEXT
1230 AE=33810
1230 AE=AE+4:NEXT
1240 PRINT:PRINT:PRINT:PRINT"OK? (Y/N)"
1240 GETB$:IFB$=""THEN2420
1260 IFB$="Y"THEN2470
1260 IFB$="N"THEN2270
1270 IFB$=CHR$(13)THEN2420
1280 GOTO2420
1290 GOTO2420
1270 RETURN
1270 RETURN
1270 REM*********** SALVAGE MENU FOR ERRORS IN TALLYING DATA *************
**PROGRAM INSTRUCTIONS**

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**PROGRAM**

```plaintext
2620 GETZ$: IFZ$="" THEN 2620
2630 IFZ$="A" THEN RESTORE: PRINT"": GOTO 270
2640 IFZ$="B" THEN END: POKE 144, 85: REM ACTIVATES R/S KEY
2650 IFZ$="C" THEN GOTO 2675
2660 IFZ$=CHR$(13) THEN 2620
2670 GOTO 2620
2675 IF R=0 THEN U%=0 = S%=0: RESTORE: PRINT"": GOTO 2680
2680 IF V=1 THEN GOTO 281
2690 V=V-1: GOTO 281
2695 REM************** PROGRAM INSTRUCTIONS **********************
2700 PRINT""
2710 PRINT" SPPF INSTRUCTIONS"
2720 PRINT" LABEL ALL CONTAMINANTS AND MISSING"
2730 PRINT" COLONIES ON THE MASTER PLATES"
2740 PRINT" AND THEIR RESPECTIVE REPLICA"
2750 PRINT" INTO SEPARATE STACKS. THEY MUST"
2760 PRINT" BE IN THE SAME RELATIVE ORDER."
2770 PRINT" DETERMINE THE TOTAL # OF"
2780 PRINT" RECOMBINANTS (EXCLUDE CONTAMINANTS"
2790 PRINT" OR MISSING COLONIES)
2800 PRINT" ENTER THE MARKERS INTO THE COMPUTER IN"
2810 PRINT" THE SAME ORDER AS THEY APPEAR ON"
2820 PRINT" CHROMOSOME MAP.
2830 PRINT" WHEN TALLYING THE DATA PRESS " FOR GROWTH AND " FOR NO GROWTH. PRESS " FOR A " CONTAMINATED OR MISSING COLONY.
2840 PRINT" TALLYING THE DATA, A MENU OF OPTIONS IS AVAILABLE IF YOU"
2850 PRINT" TO CONTINUE"
2860 GETC$: IF C$="" THEN 2860
2870 PRINT"" INSTRUCTIONS CONT."
100 PRINT"J"
105 POKE99468,12
110 PRINT"THIS IS A PROGRAM FOR PROTOPLAST"
120 PRINT"FUSION DATA ANALYSIS"
130 FORX=1 TO500:NEXT
140 PRINT"PART 2 OF THE ANALYSIS PROCEDURE"
150 FORX=1 TO1000:NEXT
160 PRINT"INSERT THE PROPER DATA DISK IN DRIVE #1"
170 PRINT"TYPE IN THE PROPER FILE NAME"
180 FD$="":PRINT
190 INPUTFD$
200 PRINT"YOU HAVE REQUESTED FILE:"
210 PRINT"";FD$;"":PRINT"DO YOU (Y/N)"
220 GETA$:IFA$=""GOTO220
230 IFA$="Y"GOTO260
240 IFA$="N"GOTO100
250 GOTO220
260 PRINT "PRESS <RETURN> WHEN DISK IS READY"
270 GETC$:IFA$=""GOTO270
280 OPEN15,8,15
290 GOSUB2440
300 OPEN1,8,3,"1: SEQ, READ"
310 PRINT"READING..."
320 INPUT#1,A,B,PK
330 CLOSE1
340 DIM(100,4),CK(100,2),X(20),REC(A/10),MM$(B),PKT$(A/10),PKT(A/10)
350 DIMPK(2A/10),RR(20,20),AC(20),RT(20),RS(20,20)
360 DIM(4),E(4),LL$(20),MR$(20),MS$(20),AA$(A/10)
370 GOSUB2440:FM$=""
380 FM$=FD$+"A"
390 OPEN3,8,4,"1: SEQ, READ"
400 FORX=1 TOB
410 INPUT#3,MM$(X)
420 NEXT
430 FORCT=1 TO PK
440 INPUT#3,PKT$(CT)
450 NEXTCT
460 FORXX=1 TO PK
470 INPUT#3,PKT(XX)
480 NEXTXX
490 GOSUB2440
500 CT=A
510 CLOSE1
515 REM SUBRT CONTROL AREA
520 GOSUB850:GOSUB1350:GOSUB260
530 GOSUB2478:GOSUB2770:GOSUB3180:END
535 REM CALCULATES MARKER FREQUENCIES
540 PRINT"WORKING..."
550 PRINT"CALCULATING MARKER FREQUENCIES..."
560 T=0
570 FORX=1 TOB:T=T+1
580 FORY=1 TO PK
590 IFMI$(PKT$(Y),T,1)="R"THENX$(X)=X$(X)+PKT(Y)
600 NEXT
610 NEXT
620 RETURN
630 REM This is the area for hard-copy print-out of all data
640 REM
650 REM
660 PRINT "I: PRINT "PART I OF THE ANALYSIS IS COMPLETE"
670 PRINT "I: PRINT "TO OBTAIN A PRINTOUT OF THE DATA"
680 PRINT "I: PRINT "TYPE IN AN EXPERIMENT LABEL SUCH AS:"
690 PRINT "I: PRINT "MYSP933297/NOVX81": PRINT "THEN HIT <RETURN>:": PRINT
700 INPUT$
710 OPEN 2,4,2
720 OPEN 1,4,1
730 OPEN 3,4
740 F$="9999.99-"
750 PRINT#2,F$
760 PRINT#3,CHR$(1)$
770 PRINT#3,
780 PRINT#3, "THE MARKER FREQUENCY AND CORRELATION DATA ARE BASED ON RECIPIENT"
790 PRINT#3, "AND RECIPIENT/RECIPIENT, DONOR/DONOR RELATIONSHIPS RESPECTIVELY"
800 PRINT#3,"BY MY COUNT YOU HAVE DATA FROM";
810 PRINT#3,"EXACTLY ":CT;"COLONIES."
820 PRINT#3,"OUT OF THESE COLONIES THE RECIPIENT"
830 PRINT#3,"MARKERS SHOWED UP LIKE THIS"
840 PRINT#3, 
850 PRINT#3,"MARKER FREQUENCY PERCENTAGE"
860 PRINT#3," REC REC DON": PRINT#3,
870 FOR X=1 TO B
880 PRINT#3,TAB(2)MM$(X);" ";
890 PRINT#1,TAB(13)X%(X);
900 PRINT#1,TAB(15)(INT(10000*(X%(X)/CT)+.5))/100;
910 PRINT#1,100-(INT(10000*(X%(X)/CT)+.5))/100
920 NEXT X
930 PRINT#3,
940 PRINT#3,"THERE WERE ":PK;" PHENOTYPES EXHIBITED"
950 PRINT#3,"": PRINT#3,
960 PRINT#3,"PHENOTYPE FREQ PERCENTAGE": PRINT#3,
970 FOR X=1 TO PK
980 PRINT#3,PKT$(X);
990 PRINT#3,PKT$(X);
1000 PRINT#1,TAB(6)PKT$(X);TAB(10)(INT(1000*PKT$(X)/CT))/10
1010 NEXT
1020 PRINT#3,"": PRINT#3," ";
1030 PRINT#3,"MARKERS": PRINT#3," ";
1040 FOR X=1 TO B: PRINT#3,A%(X);: PRINT#3," ";: NEXT
1050 PRINT#3,"CHI/DD/DR ": PRINT#3,"CHI/DD/DR ": PRINT#3,"REC";
1060 PRINT#3,
1070 P=0
1080 FOR Y=1 TO B
1090 FOR Z=1 TO B
1100 IF Y=Z THEN Q$=" ";
1110 G$=" ";Q$=" ";Z$=" ";
1120 FORS=1 TO B
1130 IF Z=S THEN Q$=Z$+MM$(S)
1140 IF Z=S THEN Q$=Q$+MM$(S)
1150 NEXT
1160 Q$=Z$+Q$
1170 PRINT#3,Q$: : PRINT#3," ";
1180 P=P+1
1190 FOR W=1 TO 4
1200 PRINT#1,A%(P,W); : NEXT
1210 PRINT#1,CX(P,1);PRINT#1,CX(P,2);
1220 CO=(AR%(P,3)+AR%(P,4))/A
1230 PRINT#1,CO;
1240 PRINT#3;
1250 NEXT
1260 NEXT
1270 CLOSE1
1280 CLOSE2
1290 CLOSE3
1300 REM***NOW RETURN TO SCREEN OUTPUT
1310 RETURN
1320 REM**************************************************************************
1330 REM THIS AREA IS WHERE 2 MARKER CROSS-OVER FREQUENCIES ARE CALCULATED
1340 REM**************************************************************************
1350 PRINT:PRINT"\\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\\t\t
1803 IF DF = 8 THEN D = 0.632: DH = 0.754
1804 IF DF = 7 THEN D = 0.666: DH = 0.798
1805 IF DF = 6 THEN D = 0.707: DH = 0.834
1806 IF DF = 5 THEN D = 0.754: DH = 0.874
1807 IF DF = 4 THEN D = 0.811: DH = 0.917
1808 IF DF = 3 THEN D = 0.878: DH = 0.959
1809 IF DF = 2 THEN D = 0.950: DH = 0.990
1810 IF DF = 1 THEN D = 0.997: DH = 1.000
1811 IF DF = 11 THEN D = 0.553: DH = 0.684
1812 IF DF = 12 THEN D = 0.532: DH = 0.661
1820 OPEN 2, 4, 2
1821 OPEN 1, 4, 1
1822 OPEN 3, 4
1823 F$ = "99999.9"
1824 PRINT#2, F$
1825 PRINT#3.
1826 PRINT#3, "MARKER COINHERITANCE FREQUENCY MATRIX": PRINT#3.
1830 PRINT#3, ";
1840 FOR X = 1 TO BB: PRINT#3, LL$(X);: PRINT#3, ";: NEXT
1845 PRINT#3.
1850 P = 0: O = 0
1860 FOR V = 1 TO BB
1865 PRINT#3, LL$(Y);: PRINT#3, ";
1870 IF MS$(Y) = "S" THEN GOSUB 2300: GOTO 1945
1875 O = O + 1
1880 FOR Y = 1 TO BB
1885 RR(X, Y) = O
1890 IF LL$(Y) = LL$(X) THEN RR(X, Y) = 100: GOSUB 2300: GOTO 1940
1895 IF MS$(Y) = "S" AND MS$(X) = "S" THEN RR(X, Y) = X(0)/CT*100: GOSUB 5000: GOTO 1940
1900 IF MS$(Y) = "R" AND RR(X, Y) = Y(0)/CT*100: GOSUB 5000: GOTO 1940
1910 IF MS$(Y) = "D" AND RR(X, Y) = D(0)/CT*100: GOSUB 5000: GOTO 1940
1920 P = P + 1
1925 RR(X, Y) = (AA%(P, 1) + AA%(P, 2))/100
1930 NEXT
1935 PRINT#3: PRINT#3.
1940 NEXT
1945 PRINT#3: PRINT#3.
1950 NEXT
1955 PRINT#3: PRINT#3.
1960 FOR Y = 1 TO BB: AB = 0
1970 FOR X = 1 TO BB
1980 AB = AB + RR(X, Y)
1990 NEXT
2000 AC(Y) = AB/BB
2010 NEXT
2020 FOR Y = 1 TO BB
2030 RT(Y) = 0
2040 FOR X = 1 TO BB
2050 RS(X, Y) = RR(X, Y) - AC(Y)
2060 RT(Y) = RT(Y) + (RS(X, Y) * RS(X, Y))
2070 NEXT
2080 NEXT
2090 CLOSE 1: CLOSE 2: CLOSE 3
2093 OPEN 2, 4, 2
2094 OPEN 1, 4, 1
2095 OPEN 3, 4
2096 F$ = "999.999-
2097 PRINT#2, F$
2098 PRINT#3:
2099 PRINT#3, "COINHERITANCE CORRELATION MATRIX": PRINT#3.
2101 PRINT#3, ";
2102 FOR X = 1 TO BB: PRINT#3, LL$(X);: PRINT#3, ";: NEXT: PRINT#3,
2090 FOR Y = 1 TO BB
2095 PRINT #3, LL$(Y); PRINT #3, " ";
2100 FOR X = 1 TO BB - RU = 0
2110 FOR Z = 1 TO BB
2120 RU = RU + (RS(Z, X) * RS(Z, Y))
2130 NEXT Z
2140 RV = RU / SQR<(RT(X) * RT(Y))) : GOSUB 7000
2150 NEXT X
2160 PRINT #3, PRINT #3,
2161 NEXT
2162 PRINT #3, " AT ", DF; " DEGREES OF FREEDOM THE CRITICAL VALUE AT ALPHA = ";
2163 PRINT #3, ".05 IS "; DG
2164 PRINT #3,
2165 PRINT #3, " AT ", DF; " DEGREES OF FREEDOM THE CRITICAL VALUE AT ALPHA = ";
2166 PRINT #3, ".01 IS "; DH
2175 CLOSE 1: CLOSE 2: CLOSE 3
2180 RETURN

2300 TT = 0
2305 FOR Z = 1 TO BB
2310 IF Z = Y THEN RR(Z, Y) = 100: GOSUB 6000: GOTO 2340
2320 IF MS$(Z) = "S" AND Z ≠ Y THEN RR(Z, Y) = 0: GOSUB 6000: GOTO 2340
2325 TT = TT + 1
2330 IF MR$(Y) = "R" THEN RR(Z, Y) = X%(TT) / CT * 100: GOSUB 6000: GOTO 2340
2335 IF MR$(Y) = "D" THEN RR(Z, Y) = (CT - X%(TT)) / CT * 100: GOSUB 6000
2340 NEXT Z: RETURN
2440 INPUT# 15, A$, B$, C$, D$
2450 IF VAL(A$) ≠ 0 THEN PRINT " EXAMINE THE RESULTS OF THE ANALYSIS"
2460 RETURN

2470 PRINT " COMPILED THUS FAR AND PROPOSE A MAP"
2480 PRINT " SEQUENCE OF THE MARKERS USED IN"
2490 PRINT " THIS EXPERIMENT."
2500 PRINT " INCLUDE ALL SELECTED MARKERS"
2510 PRINT " IMPORTANT: THE ORDER OF THE UNSELECTED MARKERS"
2520 PRINT " IN YOUR FIRST PROPOSED SEQUENCE MUST BE THE SAME"
2530 PRINT " AS THE ORDER YOU TABBED THEM INTO THE COMPUTER INITIALLY."
2540 PRINT " CHECK THE FIRST PART OF THE PRINT OUT AND INSERT THE"
2550 PRINT " SELECTED MARKERS BETWEEN THE APPROPRIATE UNSELECTED MARKERS."
2560 PRINT " IF THE ORDER OF UNSELECTED MARKERS IS NOT THE SAME,"
2570 PRINT " THE PRINTOUT WILL BE INCORRECT!!!!"
2580 PRINT " REMEMBER, THIS ONLY APPLIES TO THE FIRST PROPOSED SEQUENCE."
2590 PRINT " PRESS (RETURN) TO CONTINUE"
2600 GETA$: IF A$ = 
2610 IF A$ = 
2620 GOTO 2540
2630 GOTO 2570
2640 PRINT " ENTERING PROPOSED MARKER SEQUENCE "
2650 PRINT " "
2660 PRINT " PLEASE INPUT A 3-LETTER ABBREVIATION FOR"
2670 PRINT " EACH MARKER INCLUDE SELECTED MARKERS"
2680 PRINT " HOW MANY MARKERS ARE THERE?"
2690 INPUT X: PRINT
2691 FOR X = 1 TO BB
2692 PRINT " NUMBER" ; X ; "; IS"
2693 INPUT C$:
2694 LL$(X) = LEFT$(C$, 3)
2695 PRINT " "
2696 NEXT
2697 PRINT " "}
107

2700 PRINT"IN"; FOR X=1 TO N: PRINT": LL$(X); NEXT: PRINT"IN"
2710 PRINT"IS THIS CORRECT? (Y/N)"
2720 GETC$: IFC$="" THEN 2720
2730 IFC$="N" THEN GOTO 2470
2740 IFC$=CHR$(13) THEN 2720
2750 GOTO 2720
2760 REM******** Disting. marker as donor(D) or recipient(R)***********#
2770 PRINT"YOU MUST DESIGNATE EACH MARKER AS BEING"
2780 PRINT"FROM THE DONOR (PRESS D) OR RECIPIENT"
2790 PRINT"(PRESS R) STRAIN." : PRINT"IN"
2810 FOR X=1 TO N: PRINT" ": PRINT LL$(X); : NEXT
2820 AE=33810
2830 X=1 TO N: A$=""
2840 GETA$: IFA$="" THEN 2840
2850 IFA$="D" THEN MR$(X)=MR$(X)+A$: POKE AE,132: GOTO 2890
2860 IFA$="R" THEN MR$(X)=MR$(X)+A$: POKE AE,146: GOTO 2890
2870 IFA$=CHR$(13) THEN 2840
2880 GOTO 2840
2890 AE=AE+4: NEXT
108

3240 NEXT
3250 REM
3260 REM
3270 REM
3280 FORX=1 TO PK
3285 AA$(X)=""
3290 FORZ=1 TO BB
3300 IFMM$(Z)=LL$(Y)THEN3330
3320 GOTO3340
3330 MM$(X)=AA$(X)+MID$(PKT$(X),Z,1);Z=B
3340 NEXTZ
3350 FORY=1 TO ZZ
3360 IFY=MS(W)THEN3380
3370 COTO3390
3380 Am$(X)=Am$(X)+MR$(Y);W=ZZ
3390 NEXTW
3400 NEXTY
3410 NEXTX
3420 REM
3430 REM
3440 REM
3450 FORX=1 TO PK:REC%(X)=0:RP%(X)=0
3460 FORY=1 TO BB: EE$=""
3470 IFY=BB THENEE$=MID$(MM$(X),BB,1)+MID$(AA$(X),1,1):GOTO3490
3480 EE$=MID$(AA$(X),Y,2)
3490 IFEE$="DR" THENREC$(X)=REC$(X)+PKT(X);RP$(X)=RP$(X)+1
3500 IFEE$="RD" THENREC$(X)=REC$(X)+PKT(X);RP$(X)=RP$(X)+1
3510 NEXTY
3511 NEXTX
3512 XD%=0
3513 FORX=1 TO PK
3515 XD%=XD%+REC%(X); NEXT
3516 PRINT";"
3517 PRINT"THE TOTAL # OF CROSS-OVERS FOR YOUR"
3518 PRINT"PROPOSED SEQUENCE OF MARKERS IS #";XD%
3519 PRINT"DID YOU WANT A COMPLETE PRINTOUT? (Y/N)"
3520 GETA$:IFA$="Y"THEN3530
3521 IFA$="N"THEN3510
3522 IFA$=CHR$(13)THEN3520
3523 GOTO3520
3524 GOTO3520
3525 XD%=0
3526 FORX=1 TO PK
3527 XD%=XD%+REC%(X); NEXT
3530 OPEN2,4,2
3540 OPEN1,4,1
3550 OPEN3,4
3560 FS="99999.99-"
3570 PRINT#2,F$
3580 PRINT#3,F$
3590 PRINT#3,"THE FOLLOWING MAP SEQUENCE WAS PROPOSED...";PRINT#3,
3600 FORZ=1 TO BB
3610 PRINT#3,"X";LL$(Z);";
3620 NEXT; PRINT#3; PRINT#3,
3630 PRINT#3; PRINT#3,
3640 PRINT#3,"PHENOTYPE FREQ PERCENTAGE #X-OVERS TOTAL#OVERS"
3680 PRINT#1,TAB(6)PKT(X);TAB(10)<INT(1000*PKT(X)/CT)/10;  
3690 PRINT#3," ";  
3700 PRINT#1,RP%(X);:PRINT#3," ";  
3710 PRINT#1,REC%(X)  
3720 NEXT  
3730 PRINT#3,:PRINT#3,  
3740 PRINT#3,"THE TOTAL # OF CROSS-OVERS FOR THIS PROPOSED MAP WAS ";  
3750 PRINT#1,XD%  
3760 PRINT#3,  
3770 CLOSE1:CLOSE2:CLOSE3  
3780 ZA=ZA+1:IFZA>1THENGOTO3802  
3790 GOSUB13000  
3800 FORX=1TOPK:AA$(X)="":NEXT  
3810 FORX=1TOBB:MR$(X)="":NEXT  
3820 PRINT"**DO YOU WISH TO ANALYZE ANOTHER MAP**"  
3830 PRINT"**SEQUENCE? (Y/N)**"  
3840 GETA$:IFA$=""THEN3840  
3850 IFA$="Y"THENGOSUB2470:GOSUB2770:GOSUB2970:GOSUB3180  
3860 IFA$="N"THENRETURN  
3870 IFA$=CHR$(13)THEN3840  
3880 GOTO3840  
5000 PRINT#1,RR(X,Y);:RETURN  
6000 PRINT#1,RR(Z,Y);:RETURN  
7000 PRINT#1,RV;:RETURN

READY.