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Computer-Assisted Chromosome Mapping by Protoplast Fusion in *Staphylococcus aureus*

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Protoplasts of genetically marked derivatives of *Staphylococcus aureus* NCTC 8325 were fused with polyethylene glycol and regenerated without selection. Recombinants possessing one specific resistance marker from each parent were selected from the regenerated population and scored for seven or eight unselected markers. The results of these 9- and 10-factor crosses were entered directly into a programmed microcomputer from prescored replica plates. The data then were condensed into an array of phenotypes, together with the frequency with which each occurred. Further analyses by computer included the calculation of coinheritance frequencies for all possible pairs of markers; after entering a proposed order for the markers being analyzed, the minimum number of crossover events required to generate each phenotypic class was calculated. The linkage relationships of markers, based on the protoplast fusion data, were entirely consistent with the linkage relationships of markers already known to exist within each of the three linkage groups previously defined by transformation. The fusion data defined an arrangement of the three linkage groups into a circular chromosome map and predicted the approximate location of four previously unmapped markers (*tet-3490*, *fus-149*, *purC193*::Tn*551*, and Ω[Chr::Tn*551*]*42*) on this map.

Despite the availability of generalized transduction (24, 33) and transformation (23, 31) as methods of genetic analysis, knowledge of the genomic organization of *Staphylococcus aureus* has been limited. Transformation analyses, largely performed on the lytic group III strain 8325, resulted in the construction of three distinct linkage groups (see Fig. 1); however, it was not possible to define the relationship of these linkage groups to one another on the *S. aureus* chromosome. In addition, because of the size and complexity of the established linkage groups, mapping new markers by transformation became a laborious process. Consequently, there was interest in developing other methods of genetic exchange that might prove useful for chromosome mapping in *S. aureus*.

Genetic recombination by protoplast fusion has been described in several procaryotic species, including *Bacillus subtilis* (34), *Bacillus megaterium* (8), *Brevibacterium flavum* (20), *Escherichia coli* (36), *Providence alcalifaciens* (6), *S. aureus* (13–15), several *Streptomyces* spp. (2, 12, 18), and lactic streptococci (11). In addition to chromosomal recombination, Gotz et al. (13) demonstrated plasmid transfer among staphylococci by means of protoplast fusion. Hopwood (16) has an excellent review that includes bacterial protoplast fusions.

Protoplast fusion is unique as a mode of genetic exchange in procaryotes, because the transfer of genetic information is bidirectional and entire chromosomes are combined in the same cytoplasm at high frequencies.

The interest in protoplast fusion with *S. aureus* centered primarily on its potential as a supplementary technique for chromosome mapping. We developed a protoplast fusion procedure with *S. aureus* for the computer-assisted analysis of selected recombinant phenotypes and used it to predict the orientation of the three linkage groups on a circular map and the locations of previously unmapped chromosomal markers. The accompanying paper (35) contains data obtained by transformations with DNA extracted from protoplasts that confirm and extend the results of the fusion analysis.

MATERIALS AND METHODS

Bacteria. The strains of *S. aureus* used in this study are listed in Table 1. Some of the strains carried chromosomal insertions of Tn*551*, a transposable element that carries the *ermB*$^+$ determinant that confers constitutive erythromycin resistance (25, 27–29, 32); the *tyrB* mutation used in this study (*tyrB282*::Tn*551 ermB321*) is unable to confer erythromycin resistance

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TABLE 1. Designation, genotype, and origin of strains of *S. aureus*

Stock no.	Relevant genotype	Origin or reference
ISP2	8325 *nov-142 pig-131*	31
ISP41	8325 *nov-142 pig-131 fus-149*	Sp × ISP2[a]
ISP95	Ps47 *tet-3490*	Asheshov[b]
ISP193	8325 *thy-101 thrB106 ilv-129 pig-131* Ω(Chr::Tn*551*)*11* $\phi 12^-$	30
ISP267	8325 *nov-142 rib-127 pig-131 tmn-3106*	30
ISP479	8325-4 (pI258 *bla-401 mer-14 repA36*) *pig-131*	29
ISP483	8325 *uraA141 hisG15 nov-142 mec-4916 pig-131*	30
ISP540	8325-4 *pur-190*::Tn*551 pig-131*	43C × ISP479[c]
ISP542	8325-4 *purC193*::Tn*551 pig-131*	43C × ISP479
ISP794	8325 *pig-131*	ISP2 DNA × ISP1[d,e]
ISP796	8325 *pig-131* Ω(Chr::Tn*551*)*34*	RN2573 DNA × ISP794
ISP797	8325 *pig-131 purC193*::Tn*551*	ISP542 DNA × ISP794
ISP803	8325 *pig-131* Ω(Chr::Tn*551*)*42*	RN1857 DNA × ISP794
ISP808	8325 *pig-131 pur-190*::Tn*551*	ISP540 DNA × ISP794
ISP933	8325 *thrB106 uraA141 ilv-129 mec-4916 nov-142 pig-131 ala-126 tmn-3106 trpE85 tyrB282*::Tn*551 ermB321*	ISP839 DNA × ISP930[d]
ISP983	8325 *thrB106 uraA141 ilv-129 mec-4916 nov-142 pig-131* Ω(Chr::Tn*551*)*5 trpE85 tyrB282*::Tn*551 ermB321*	RN496 DNA × ISP933
ISP988	8325 *thrB106 trpE85 tyrB282*::Tn*551 ermB321 ilv-129 pig-131 uraA141 nov-142 mec-4916 tmn-3106*	ISP267 DNA × ISP983
ISP1008	8325 *nov-142 pig-131 fus-149 tet-3490*	ISP1038 DNA × ISP41
ISP1038	8325 *uraA141 hisG15 nov-142 mec-4916 pig-131 tet-3490*	ISP95 DNA × ISP483
RN496	8325-4 Ω(Chr::Tn*551*)*5 pig-131*	28
RN1857	8325 Ω(Chr::Tn*551*)*42 pig-131*	Novick[f]
RN2573	8325 (80α) Ω(Chr::Tn*551*)*34 pig-131*	30; Novick[f]

[a] Spontaneous fusidic acid-resistant mutant of strain ISP2.
[b] E. H. Asheshov, Central Public Health Laboratory, London, England.
[c] Isolated by Tn*551* mutagenesis by the method of Pattee (29).
[d] Detailed origin given in Pattee et al., submitted for publication.
[e] Strain ISP1 was transformed with DNA taken from strain ISP2.
[f] Richard P. Novick stock culture collection, Department of Plasmid Biology, The Public Health Research Institute of the City of New York, Inc., New York, N.Y.

because of a point mutation (*ermB321*) within Tn*551* (Pattee et al., submitted for publication). Pattee (29) provides a detailed description of Tn*551* insertion mutagenesis. All cultures were maintained on brain heart infusion (Difco Laboratories) agar slants stored at 4°C. A second set of stock cultures was maintained at −70°C in GL broth (28) plus 10% glycerol.

Media and reagents. All dehydrated commercial media were supplemented with thymine (20 μg/ml), adenine, guanine, cytosine, and uracil (each at 5 μg/ml) (31). The composition of complete defined synthetic (CDS) agar was modified by omitting the appropriate amino acids, purines, and pyrimidines and adding antibiotics as needed (5, 30). Antibiotic resistance phenotypes were selected by adding the appropriate concentration of antibiotic to brain heart infusion agar. The majority of the genetic markers used in this study have been described (5, 21, 29, 30). An auxotrophic marker affecting L-alanine biosynthesis (*ala-126*) was scored on L-alanine-deficient CDS agar. Resistance to fusidic acid, imposed by *fus-149*, was scored on 10 μg of fusidic acid per ml. The *ermB321* mutation that impaired resistance to erythromycin by the *ermB*$^+$ marker was scored on 10 μg of erythromycin per ml.

Protoplasts were formed in sucrose-magnesium-Tris buffer (SMTB; 100 mM Tris, 40 mM $MgSO_4$, 0.8 M sucrose, pH 7.6). DNase I (Sigma Chemical Co.) stock solution (3 mg/ml) was dissolved in 0.005 M $MgSO_4$. Lysostaphin (Sigma) was dissolved at 1 mg/ml in 600 mg of Tris–870 mg of NaCl–100 ml of deionized water at pH 7.5. The DNase and lysostaphin stocks were filter sterilized and stored in 1-ml portions at −20°C. Protoplasts were fused in 60% (vol/vol) polyethylene glycol (PEG; molecular weight, 400; Sigma) in SMTB. Regeneration (R) medium consisted of Trypticase soy broth (BBL Microbiology Systems), 30 g; sucrose (Sigma), 273 g; agar (Difco), 25 g; sodium citrate, 0.5 g; starch, 2.1 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 of medium per 15- by 100-mm plate, were dried overnight at 35°C before use.

Protoplast fusion procedure. Parental cells harvested in saline (0.85% NaCl) from overnight brain heart infusion agar slants were inoculated into 100-ml volumes of Trypticase soy broth in 300-ml nephelometer flasks to an optical density at 540 nm of 0.1. The cultures were shaken gently at 35°C until an optical density of 0.65 (late-log-phase cells) was reached. Because the parental strains had different growth rates, each strain was inoculated into Trypticase soy broth so that the cells from both cultures could be harvested simultaneously at the desired optical density. The cells were harvested by centrifugation (10,000 × *g*, 25 min, 4°C) and washed once in saline. The cells of each parent strain from 200 ml of Trypticase soy broth were suspended in 10 ml of SMTB containing

DNase (15 μg/ml) and lysostaphin (30 μg/ml) and transferred to 50-ml screw-capped Erlenmeyer flasks. The flasks then were turned gently 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 × 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 was then gently but thoroughly mixed and incubated in a circulating water bath without shaking at 20°C for 1 min. Samples (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 to 80% relative humidity) for 7 days.

Genetic analysis. Growth from five R medium plates was collected in saline (5 ml per 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 the cell suspensions were pooled, diluted samples were spread on brain heart infusion agar to assay the number of CFU. Samples of the diluted and undiluted suspensions were also spread onto the appropriate selective media. After incubation at 35°C for 48 h, approximately 600 isolated colonies from each selected class were picked to the same selective medium to form master plates, each with an ordered array of 56 recombinants. After 24 h at 35°C, each master plate was velveteen-replicated onto a fresh plate of the same medium, which was then incubated for another 24 h at 35°C. This step insured that cells from each clone would carry over in sufficient numbers to each of as many as nine replica plates. These second masters were velveteen-replicated to appropriate media to score the unselected phenotypes among the recombinants in the sequence Ilv (isoleucine-valine), Thr (threonine), Trp (tryptophan), Ala (alanine), Tyr (tyrosine), Ura (uracil), Tmn (tetracycline or minocycline) or Tet (tetracycline), Em (erythromycin), Nov (novobiocin), Fus (fusidic acid), and Mec (methicillin). After incubation at 35°C for 24 or 48 h the replicas were examined, and those plates that had clearly differentiated growth responses were prescored and held at room temperature. All plates selective for the same marker were then arranged in stacks in a sequential order identical to the labeled sequence of the master plates. Contaminants and colonies missing from the ordered array of 56 colonies per plate were clearly marked.

The controls incorporated into each experiment included the following modifications of the fusion procedure. (i) Parent cells received SMTB instead of lysostaphin during the preparation of protoplasts, so that normal cells of each parent were subjected to the fusion procedure (cell controls). (ii) SMTB was substituted for the PEG solution during the fusion procedure, so that only spontaneous fusion events would be observed (fusion controls). (iii) PEG solution was added to a double volume of protoplasts from a single parent (two of these controls were required per experiment) (reversion controls). All controls were harvested and assayed for CFU and recombinants in the same manner as for the experimental plates before growth response data were entered into the computer.

Analysis of data. A programmed microcomputer (CBM model 8032 computer, model 8050 dual disk drive, printer; Commodore Computer Systems, Wayne, Pa.) was used to record and analyze the results of fusion experiments. The program first required entry of the number of unselected markers being analyzed, the parent from which each allele originated, and the total number of recombinants being scored. Beginning with the first plate containing 56 recombinants scored for the first marker, the growth response (+ or −) of each recombinant was entered at the keyboard; a video display of each colony in the pattern used on the plates (which visually recorded each entry) greatly facilitated the entry of these data, which were stored into a two-dimensional matrix that ultimately contained the phenotype of each recombinant. From these data, each pair of markers (including the selected markers) was assigned a coinheritance frequency (CIF). The CIF for a specific pair of markers was defined as the percentage of the total number of recombinants that had either parental phenotype for that specific pair of markers. After a proposed order for the markers in a fusion experiment was entered into the computer, the minimum number of crossover events required to account for each phenotype (minimum of two) was calculated, together with the total number of crossovers for the experiment (theoretical minimum of two times the number of recombinants analyzed). A copy of the fusion program on disk (DOS 2.5) or tape is available from one of us (P.A.P.).

RESULTS

Recombinants were readily detected when recombination was measured by velveteen-replicating the growth directly from R medium onto media selective for various pairs of parental markers. Because these regenerated clones were always heterogeneous for unselected markers, this method was inappropriate for accurately measuring the frequency of genetic recombination leading to stable haploid recombinants. In contrast, when the cells were harvested from R medium, disaggregated by sonication, and respread on selective media, the cells in any one of the recombinant clones so obtained were always homogeneous for unselected markers, although the distribution of unselected markers differed from one clone to another. Using this procedure and strains ISP193 and ISP483, we examined the optimum conditions for protoplast fusion. The effects of culture age, duration of exposure of the parental cells to lysostaphin, the molecular weight (200, 400, or 1,000) and concentration of PEG, the presence of dimethyl sulfoxide in the fusion mixture, and some nutritional and physical aspects of regeneration on recombination frequencies were examined. The fusion protocol described above was based on these experiments and gave the highest recombination frequencies of any examined. The details of these experiments, and hard copy of the computer program for fusion analysis, have been published (M. L. Stahl, Ph.D. dissertation, Iowa State University, Ames, 1982).

Experimental reproducibility. Because the ma-

TABLE 2. Experimental reproducibility of recombination frequencies and distribution of unselected markers among selected classes

Selected class[a]	Recombination frequency (%)[b]	% of recombinants (SD)[c] that were:	
		Ilv⁻	Thr⁻
Thy⁺ His⁺	3.6×10^6 (44)	49.5 (2.3)	49.2 (2.3)
Thy⁺ His⁺ Em^r	5.8×10^5 (52)	84.5 (4.4)	43.2 (4.6)

[a] Em^r = Ω(Chr::Tn*551*)*11*.

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

[c] Mean results from four fusion experiments (strains ISP1943 × ISP483).

jor interest in protoplast fusion was its use for chromosome mapping, the reproducibility of the fusion data was evaluated by simple statistical analysis of the results from four identical fusions between strains ISP193 and ISP483. The results (Table 2) showed that although the recombination frequencies varied by ±50%, the distribution of unselected markers among the recombinants varied by only about 5%.

Fusion controls. When fusions between parents of the same phage-typing pattern (reflecting the same prophage content) were performed (see Tables 3 through 7), between 1 and 50 revertants per 10^9 CFU were recovered from all controls. These reversion frequencies were consistent with the spontaneous reversion rates of the selected markers; therefore, revertants did not contribute to the recombinants analyzed from fusion mixtures because of the dilution required to recover isolated recombinants. When parents known to differ in their prophage content were fused, the cell and fusion controls (but not the reversion controls) often yielded as many as 10^3 revertants per 10^9 CFU. This higher reversion frequency, seen only when the parents (as cells or protoplasts) were plated together, may reflect transduction among the parents.

Protoplast fusion analyses. Many early experiments made use of selection for three or more markers, some of which were auxotrophs. Selection for recombinants that had acquired a resistance marker and one or more prototrophic markers was inefficient and, in some cases, totally unsuccessful, probably because of the necessity of using CDS rather than brain heart infusion agar for selection. Therefore, two antibiotic resistance determinants (one from each parent) were used for selection in all future experiments. Selection exclusively for antibiotic resistance determinants was most consistent, because background growth (consisting of parental and unselected recombinant phenotypes) was suppressed adequately by the presence of antibiotics in the medium. In selections for prototrophic recombinants, 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 because many of the clones on the master plates were not pure cultures of the desired recombinant phenotype.

Crosses between parent strains that differed in their prophage patterns often resulted in recombinants with a mottled colony morphology indicative of lytic infection. To avoid this problem, we used two distinct classes of parent strains in all subsequent fusions. One class consisted of multiply marked strain 8325 derivatives with eight or nine chromosomal markers; these strains included ISP933, ISP983, and ISP988 (Table 1). The second class consisted of a series of strain ISP794 derivatives that contained the marker of interest. The marker of interest was usually a chromosomal Tn*551* insertion that originated from strain ISP479; before analysis by fusion, these Tn*551* insertions were transformed into strain ISP794. Some of the Tn*551* insertions occupied chromosomal sites of interest, such as the known ends of the linkage groups (Fig. 1); others were not within the known linkage groups and were designated orphan insertions.

The experimental parameters of fusion analysis were developed to most nearly attain conditions under which the exchange of genetic information was completely random and bidirectional. To determine how successfully this objective was met, we performed a fusion between strains ISP933 and ISP808. After regeneration (in the absence of selection), the cells were harvested from the R medium, sonicated, diluted, and inoculated onto brain heart infusion agar without selection. Among 1,232 colonies analyzed, 6% were recombinant, 69% were strain ISP808 phenotype, and 25% were strain ISP933 phenotype. Among the 77 recombinants, there were 25 distinct phenotypes, within which the occurrence of each ISP933 marker was: Thr⁻, 73%; Trp⁻, 69%; Tyr⁻, 18%; Tmn^r, 38%; Ilv⁻, 38%; Ura⁻, 56%; Nov^r, 68%; and Mec^r, 68%. The distribution of the 166 crossovers required to account for the recombinant phenotypes (assuming the marker order to be as listed above and circular) was: Thr-Trp, 7.8%; Trp-Tyr, 27%; Tyr-Tet, 10.2%; Tet-Ilv, 15.1%; Ilv-Ura, 10.2%; Ura-Nov, 6.6%; Nov-Mec, 1.2%; and Mec-Thr, 21.6%. Thus, under conditions intended to allow random genetic recombination and regeneration, the incidence of crossover events was reasonably random. The abnormally high incidence (82%) of Tyr⁺ recombinants represented but one

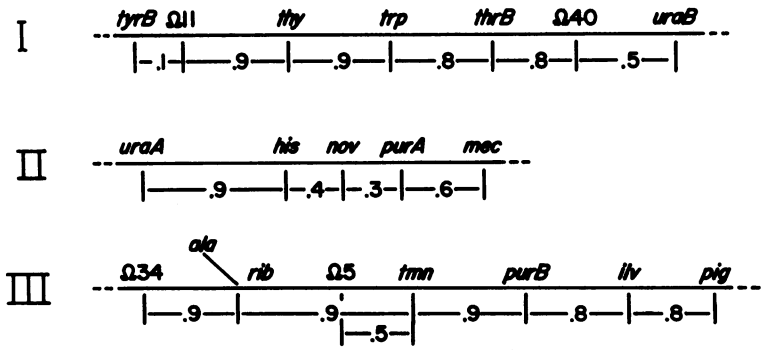

FIG. 1. The three known linkage groups of the chromosome of *S. aureus* NCTC 8325. Only markers pertinent to the present study are shown. Map distances are averages of several experiments and are expressed as the estimated cotransformation frequency subtracted from 1 (redrawn with revisions from reference 29).

example of the poor recovery of auxotrophic alleles among recombinants; it was responsible for the low CIFs for Tyr with Trp (to which it is known to be linked) in the results presented below.

The relative map position of Ω(Chr::Tn*551*)*42*, an orphan Tn*551* insertion, was examined by fusing strains ISP933 and ISP803 (Table 3). Pairs of markers known, by transformation, to be linked (Fig. 1) usually exhibited high CIFs; although this was most obvious for the Ura, Nov, and Mec markers of linkage group II (CIF > 92%), it also was apparent for linkage groups I and III. An exception was *ala-126*, known from transformation data (Fig. 1) to be adjacent to *tmn-3106*; this was attributed largely to the very poor survival or regeneration or both of Ala$^-$ recombinants. In the experiment summarized in Table 3, either 93% (Em^r Nov^r selection) or 89% (Em^r Tmn^r selection) of the recombinants were Ala$^+$. The low incidence of Ala$^-$ recombinants was also reflected in the disproportionately large number of crossovers observed (cf. footnotes *c* and *d*, Table 3), which were 170 to 190% of the theoretical minimum. Also, among the 616 Em^r Tmn^r recombinants, 448 (73%) experienced either four or six crossovers, two of which occurred between Ala and the markers adjacent to it. Strains ISP988 and ISP983, constructed from strain ISP933 and lacking *ala-126*, were used for most other fusion experiments. Interpretation of the results (Table 3) placed Ω(Chr::Tn*551*)*42* to the left of *thrB106* in linkage group I. Also, the CIFs (78 and 84%, respectively; Table 3) for Ilv and Ura suggested that these markers (and thus the linkage groups containing them) were linked.

The map location of *purC193*::Tn*551*, an orphan insertion, was predicted from the CIFs

TABLE 3. CIFS for a strain ISP933 × ISP803[a] protoplast fusion

Phenotype	CIF (%)[b]																			
	Em^r Tmn^r recombinants[c]										Em^r Nov^r recombinants[d]									
	Em	Thr	Trp	Tyr	Ala	Tmn	Ilv	Ura	Nov	Mec	Em	Thr	Trp	Tyr	Ala	Tmn	Ilv	Ura	Nov	Mec
Em	100	74	61	17	89	0	19	32	33	34	100	84	75	38	93	34	25	6	0	1
Thr	74	100	84	37	68	26	30	34	32	33	84	100	88	48	79	44	37	20	16	17
Trp	61	84	100	49	56	39	37	40	38	39	75	88	100	56	70	51	44	28	25	26
Tyr	17	37	49	100	7	83	72	59	62	61	38	48	56	100	32	89	79	65	61	62
Ala	89	68	56	7	100	11	23	36	33	33	93	79	70	32	100	40	30	13	7	7
Tmn	0	26	39	83	11	100	81	68	67	66	34	44	51	89	40	100	89	69	66	66
Ilv	19	30	37	72	23	81	100	84	83	82	25	37	44	80	30	89	100	78	75	75
Ura	32	34	40	59	36	68	84	100	96	95	6	20	28	65	13	69	78	100	94	93
Nov	33	32	38	62	33	67	83	96	100	99	0	16	25	61	7	66	75	94	100	99
Mec	34	33	39	61	33	66	82	95	99	100	1	17	26	62	7	66	75	93	99	100

[a] Phenotypes: strain ISP933, Em^s Thr$^-$ Trp$^-$ Tyr$^-$ Ala$^-$ Tmn^r Ilv$^-$ Ura$^-$ Nov^r Mec^r; strain ISP803, Em^r Thr$^+$ Trp$^+$ Tyr$^+$ Ala$^+$ Tmn^s Ilv$^+$ Ura$^+$ Nov^s Mec^s. Em^r = Ω(Chr::Tn*551*)*42*.

[b] The numbers represent the percentages of the total number of recombinants with either of the parental phenotypes for the indicated pairs of markers.

[c] The recombination frequency was 3.4×10^6 per 10^9 CFU; 616 recombinants scored yielded 40 phenotypes requiring at least 2,348 crossovers.

[d] The recombination frequency was 3.9×10^6 per 10^9 CFU; 616 recombinants scored yielded 37 phenotypes requiring at least 2,090 crossovers.

TABLE 4. CIFs for a strain ISP933 × ISP797[a] protoplast fusion

Phenotype	CIF (%)[b]																	
	Em[r] Tmn[r] recombinants[c]									Em[r] Nov[r] recombinants[d]								
	Em	Thr	Trp	Tyr	Tmn	Ilv	Ura	Nov	Mec	Em	Thr	Trp	Tyr	Tmn	Ilv	Ura	Nov	Mec
Em	100	79	78	35	0	10	18	18	18	100	85	82	69	57	38	6	0	0
Thr	79	100	98	40	21	29	30	30	30	85	100	96	66	57	41	19	15	15
Trp	78	98	100	41	22	29	31	31	31	82	96	100	66	57	41	21	18	18
Tyr	35	40	41	100	65	71	72	72	72	69	66	66	100	85	68	37	31	31
Tmn	0	21	22	65	100	90	82	82	82	57	57	57	85	100	81	49	43	43
Ilv	10	29	29	71	90	100	92	92	92	38	41	41	67	81	100	67	62	62
Ura	18	30	31	72	82	92	100	99	99	6	19	21	37	49	67	100	94	94
Nov	18	30	31	72	82	92	99	100	100	0	15	18	31	43	62	94	100	100
Mec	18	30	31	72	82	92	99	100	100	0	15	18	31	43	62	94	100	100

[a] Phenotype: strain ISP933, Em[s] Thr[-] Trp[-] Tyr[-] Tmn[r] Ilv[-] Ura[-] Nov[r] Mec[r]; strain ISP797, Em[r] Thr[+] Trp[+] Tyr[+] Tmn[s] Ilv[+] Ura[+] Nov[s] Mec[s]. Em[r] = *purC193*::Tn*551*.

[b] The numbers represent the percentages of the total number of recombinants with either of the parental phenotypes for the indicated pairs of markers.

[c] The recombination frequency was 1.8×10^6 per 10^9 CFU; 616 recombinants scored yielded 23 phenotypes requiring at least 1,342 crossovers.

[d] The recombination frequency was 4.8×10^6 per 10^9 CFU; 616 recombinants scored yielded 22 phenotypes requiring at least 1,392 crossovers.

(Table 4) to be linked to the *thrB106-trpE85* marker pair in linkage group I. The high CIFs for both selections were consistent with pairs of markers known to be linked. Crossovers were particularly rare between *thrB106* and *trpE85*, as CIFs for these selected classes were greater than 95%. Also, the Tmn-Ilv (CIF, 81 or 90%) and the Ura-Nov-Mec (CIF, 94 or 99%) linkages were coinherited at high frequency. Although strain ISP933 carried *ala-126*, the Ala phenotype was not scored in this analysis.

An attempt was then made to detect close linkages among markers on the ends of the known linkage groups by fusing strains ISP988 and ISP796. The Ω(Chr::Tn*551*)*34* marker was chosen for use in this experiment because of its location on the left extremity of linkage group III (Fig. 1) and its weak linkage by transformation to the *tmn-3106* locus. The CIFs (Table 5) revealed a new linkage between TyrB and Em (CIF, 98 or 99%). Because *tyrB282*::Tn*551 ermB321* occupied the extreme right end of linkage group I, these results strongly indicated that linkage groups I and III were adjacent on the chromosome, with *tyrB282* and Ω(Chr::Tn*551*)*34* being proximal.

TABLE 5. CIFs for a strain ISP988 × ISP796[a] protoplast fusion

Phenotype	CIF (%)[b]																	
	Em[r] Tmn[r] recombinants[c]									Em[r] Nov[r] recombinants[d]								
	Thr	Trp	Tyr	Em	Tmn	Ilv	Ura	Nov	Mec	Thr	Trp	Tyr	Em	Tmn	Ilv	Ura	Nov	Mec
Thr	100	92	35	36	64	61	66	65	65	100	90	41	41	47	54	59	59	59
Trp	92	100	41	41	59	56	60	60	60	90	100	37	36	41	54	64	63	63
Tyr	35	41	100	98	2	12	9	9	9	41	37	100	99	73	50	6	1	1
Em	36	41	98	100	0	10	7	7	8	41	36	99	100	72	49	4	0	0
Tmn	64	59	2	0	100	90	93	92	92	47	41	73	72	100	76	32	28	28
Ilv	61	56	12	10	90	100	92	92	92	53	54	50	49	76	100	55	51	51
Ura	66	60	9	7	93	92	100	99	99	60	64	6	4	32	55	100	96	96
Nov	65	60	9	7	92	92	99	100	99	59	63	1	0	28	51	96	100	100
Mec	65	60	9	8	92	92	99	99	100	59	63	1	0	28	51	96	100	100

[a] Phenotype: strain ISP988, Thr[-] Trp[-] Tyr[-] Em[s] Tmn[r] Ilv[-] Ura[-] Nov[r] Mec[r]; strain ISP796, Thr[+] Trp[+] Tyr[+] Em[r] Tmn[s] Ilv[+] Ura[+] Nov[s] Mec[s]. Em[r] = Ω(Chr::Tn*551*)*34*.

[b] The numbers represent the percentages of the total number of recombinants with either of the parental phenotypes for the indicated pairs of markers.

[c] The recombination frequency was 2.9×10^6 per 10^9 CFU; 504 recombinants scored yielded 20 phenotypes requiring at least 1,124 crossovers.

[d] The recombination frequency was 1.7×10^7 per 10^9 CFU; 504 recombinants scored yielded 19 phenotypes requiring at least 1,092 crossovers.

TABLE 6. CIFs for a strain ISP983 × ISP95[a] protoplast fusion

Phenotype	CIF (%)[b]																		
	Em^r Tet^r recombinants[c]									Nov^r Tet^r recombinants[d]									
	Thr	Trp	Tyr	Em	Ilv	Ura	Nov	Mec	Tet	Thr	Trp	Tyr	Em	Ilv	Ura	Nov	Mec	Tet
Thr	100	92	45	27	36	75	77	78	72	100	93	68	59	59	60	59	69	41
Trp	92	100	47	29	39	77	77	77	71	93	100	73	63	63	64	63	72	37
Tyr	45	47	100	72	68	39	38	37	28	68	73	100	73	73	75	73	81	27
Em	27	29	72	100	82	16	12	10	0	59	63	73	100	99	98	99	82	1
Ilv	36	39	68	82	100	34	30	29	18	59	63	73	99	100	98	99	83	1
Ura	75	77	39	16	34	100	96	95	84	60	64	75	98	98	100	98	81	2
Nov	77	77	38	12	30	96	100	98	88	59	63	73	99	99	98	100	83	0
Mec	78	77	37	10	29	95	98	100	90	69	72	81	82	83	81	83	100	17
Tet	72	71	28	0	18	84	88	90	100	41	37	27	1	1	2	0	17	100

[a] Phenotype: strain ISP983, Thr$^-$ Trp$^-$ Tyr$^-$ Em^r Ilv$^-$ Ura$^-$ Nov^r Mec^r Tet^s; strain ISP95, Thr$^+$ Trp$^+$ Tyr$^+$ Em^s Ilv$^+$ Ura$^+$ Nov^s Mec^s Tet^r. Em^r = Ω(Chr::Tn*551*)*5*.

[b] The numbers represent the percentages of the total number of recombinants with either of the parental phenotypes for the indicated pairs of markers.

[c] The recombination frequency was 4.5×10^5 per 10^9 CFU; 560 recombinants scored yielded 28 phenotypes requiring at least 1,210 crossovers.

[d] The recombination frequency was 1.1×10^5 per 10^9 CFU; 448 recombinants scored yielded 17 phenotypes requiring at least 1,006 crossovers.

With linkage groups I and III linked, it was only necessary to determine the proper orientation of linkage group II to define a circular map of the chromosome. The order was determined by fusing strains ISP95 and ISP983. Strain ISP95 carries *tet-3490*, a tetracycline resistance determinant that was not within the known linkage groups. The CIF matrix for the Em^r Tet^r recombinants (Table 6) showed high CIFs for Tet and the markers in linkage group II: Mec at 90%, Nov at 88%, and Ura at 84%. Tet also coinherited at high frequencies with Thr (72%) and Trp (71%). Additional evidence for a *mec-4916–tet-3490–thrB106* linkage is shown in the CIF matrix for the Nov^r Tet^r recombinants (Table 6). Among the Nov^r Tet^r recombinants, 17% had experienced a crossover between Nov and Mec, but only 2% had a crossover between Nov and UraA. In previous fusion experiments, an average of 1 or 2% of the selected Nov^r recombinants had experienced crossovers between Nov and Ura or between Nov and Mec. The orientation of the Ura-Nov-Mec determinants relative to Ilv and Tet was evident in the minimum number of crossovers required to account for the phenotypes observed. Among 560 Em^r Tet^r recombinants, 28 phenotypes were observed. The order Ilv-Ura-Nov-Mec-Tet required 1,210 crossovers, whereas 1,298 crossovers would be required if the marker order was Ilv-Mec-Nov-Ura-Tet. Among 448 Nov^r Tet^r recombinants (17 phenotypes observed), these values were 1,006 and 1,140, respectively. The CIF for Tet and Thr was 41%, the highest observed between Tet and any other marker in the cross. The low coinheritance of Tet with other markers can be explained by the high percentage (46%) of recombinants in which *tet-3490* was the only strain ISP95 marker inherited; only 7.5% of the Em^r Tet^r recombinants had this phenotype.

A fusion of strains ISP983 and ISP1008 (Table 7) confirmed the orientation of the Ura-Nov-Mec linkage group and the location of *tet-3490* between *thrB106* and *uraA141* and determined the approximate location of a spontaneous fusidic acid resistance determinant, *fus-149*. The high CIF for Tet-Fus in both classes of recombinants (88 and 92%) placed these two markers together, and their CIFs with the remaining markers placed Tet and Fus between Thr and Mec. Ura and Mec coinherited with Ilv, Tet, Fus, and Thr in a manner consistent with Mec being proximal to the Tet-Fus pair and Ura being adjacent to Ilv.

The results of the fusion experiments described in this report allowed the three linkage groups previously defined by transformation to be arranged into a circular chromosome map (Fig. 2). The approximate locations of the *tet-3490*, *fus-149*, *purC193*::Tn*551* and Ω(Chr::Tn*551*)*42* also are shown.

DISCUSSION

Several factors influenced the successful preparation, fusion, and regeneration of protoplasts of *S. aureus*. Because nonprotoplasted cells inhibit the regeneration of protoplasts, high efficiencies of protoplast formation were sought even at the expense of survival of some of the protoplasts. Surviving walled cells (defined by their colony-forming ability on brain heart infusion agar lacking sucrose) were held to less than 1 cell per 10^9 protoplasts. This efficiency was attained with 22% of the original cells surviving

TABLE 7. CIFs for a strain ISP983 × ISP1008[a] protoplast fusion

Phenotype	CIF (%)[b]																
	Em[r] Fus[r] recombinants[c]									Em[r] Tet[r] recombinants[d]							
	Tet	Fus	Thr	Trp	Tyr	Em	Ilv	Ura	Mec	Tet	Fus	Thr	Trp	Tyr	Em	Ilv	Ura	Mec
Tet	100	88	82	82	38	12	31	81	84	100	92	94	90	23	0	15	69	80
Fus	88	100	92	93	33	0	19	69	72	92	100	90	88	29	8	22	68	73
Thr	82	92	100	94	37	8	24	67	68	94	90	100	93	25	6	21	69	74
Trp	82	93	94	100	36	7	24	67	69	90	88	93	100	29	10	24	70	76
Tyr	38	33	37	36	100	67	60	45	42	23	29	25	29	100	77	71	37	33
Em	12	0	8	7	67	100	81	31	28	0	8	6	10	77	100	85	31	20
Ilv	31	19	24	24	60	81	100	49	45	15	22	21	24	71	85	100	45	34
Ura	81	69	67	67	45	31	49	100	91	69	68	69	70	37	31	45	100	87
Mec	84	72	68	69	42	28	45	91	100	80	73	74	76	33	20	34	87	100

[a] Phenotype: strain ISP983, Tet[s] Fus[s] Thr[-] Trp[-] Tyr[-] Em[r] Ilv[-] Ura[-] Mec[r]; strain ISP1008, Tet[r] Fus[r] Thr[+] Trp[+] Tyr[+] Em[s] Ilv[+] Ura[+] Nov[s]. Em[r] = Ω(Chr::Tn*551*)5.

[b] The numbers represent the percentages of the total number of recombinants with either of the parental phenotypes for the indicated pairs of markers.

[c] The recombination frequency was 3.7×10^5 per 10^9 CFU; 672 recombinants scored yielded 39 phenotypes requiring at least 1,468 crossovers.

[d] The recombination frequency was 4.1×10^5 per 10^9 CFU; 672 recombinants scored yielded 42 phenotypes requiring at least 1,488 crossovers.

as protoplasts and was possible only by rolling the cells during lysostaphin treatment rather than by reciprocal shaking. The addition of 2.5% agar and 0.3% starch to R medium resulted in a 10- and 20-fold increase in regeneration frequency, respectively, and yielded an average regeneration frequency of about 1.0%, based on Petroff-Hausser chamber counts of protoplasts before fusion. The use of high agar concentrations reduces the diffusion of substances produced by early-developing colonies arising from surviving nonprotoplasted cells that inhibit regeneration (7). This problem was encountered in other systems (7, 18, 22) and was attributed to autolysins in *B. subtilis*; extracellular lipases or hemolysins may be responsible in *S. aureus*. The enhancement of regeneration by starch may be related to the similar effects of serum and gelatin in *B. subtilis* (1). The maintenance of artificially high humidity during regeneration also was essential in obtaining consistent results; this is in contrast to the results of Baltz and Matsushima (4), who found that considerable dehydration of the base agar layer stimulated regeneration of *Streptomyces* protoplasts.

Fodor and Alfoldi (9), in studying fusions with *B. megaterium*, 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. This variation probably was caused by their use of minimal medium for growing the cells for protoplast formation and the application of selective pressures during regeneration. In contrast, Schaeffer et al. (34), working with *B. subtilis*, and Hopwood and Wright (17), using *Streptomyces coelicolor*, grew the parent strains for protoplast formation in complex media, and regeneration was nonselective; both groups obtained linkage relationships that were consistent with the known linkage maps. Baltz (3) defined a linkage map of *Streptomyces fradiae* based on fusion analysis by arranging the markers involved in the cross so that the incidence of multiple crossovers was minimal.

The primary objectives of this study were to develop fusion analysis as a supplementary technique for chromosome mapping in *S. aureus* and to define the arrangement of the established linkage groups on a circular map. Therefore, very high frequencies of regeneration and recombination were less important than were experimental conditions that allowed random exchange of genetic information. Under these circumstances, the coinheritance of any two markers from either parent would most nearly be a function of their relative positions on the parental chromosomes. In 9- and 10-factor fusion crosses from which specific classes of recombinants were selected, between 10^{-5} and 10^{-2} recombinants were recovered per viable cell. When a random sample of cells from R medium was scored, approximately 6% of the entire population had experienced at least two crossovers that were detected among the markers examined. Although the recombination frequencies varied ±50%, the distribution of unselected markers was reproducible to within ±5% (Table 2). Thus, the CIFs were reasonably reproducible values that should reflect the relative distances separating marker pairs. The validity of this assumption was confirmed by the results of this and the accompanying paper (35).

Even under conditions intended to support random and bidirectional exchange of genetic information, the recovery of recombinants favored those that had inherited the fewest auxotrophic markers. This conclusion was supported by the results of the ISP933 × ISP808 fusion, in which the parents and recombinants were recovered in the absence of selection. Among the 1,232 clones isolated under nonselective conditions, 94% were nonrecombinant; 75% of these were strain ISP808 phenotype (the prototrophic parent), and only 25% were strain ISP933 phenotype (the multiply marked parent). Although equivalent numbers of cells from the two parents were used in this experiment, the disproportionate number of strain ISP808 phenotype clones recovered may reflect many of the experimental parameters that adversely effect strain ISP933, including survival as viable protoplasts, regeneration efficiency, and the growth rate of the descendants of the protoplasts after regeneration.

The recombinants obtained by protoplast fusion were assumed to be stable haploids because the phenotypes of fusion recombinants were stable after repeated subculturing and they acted as true haploids when used as recipients or as sources of donor DNA in transformations (unpublished data). Also, the biparentals or diploids that were isolated from *B. subtilis* protoplast fusions by Hotchkiss and Gabor (19) were noncomplementary; consequently, the diploids were not capable of growing on media selective for recombinant phenotypes.

The most difficult and time-consuming aspect of developing fusion analysis for chromosome mapping in *S. aureus* was the construction of multiply marked parent strains with useful markers in strategic map locations. Related to the development of these parent strains was the necessity of using parent strains that were isogenic in their prophage content. In many early fusions, recombinants growing on selective media showed the mottled colony morphology characteristic of lytically infected recombinants. This difficulty was caused by heterogeneities in the prophage patterns of the parent strains. Many of the markers for which map locations were sought originated in strain ISP479, a plasmid-bearing derivative of strain 8325-4; strain 8325-4 lacks prophages ϕ11, ϕ12, and ϕ13, which are native to strain 8325 (26). Successful fusion experiments were possible only after these markers were moved into strain ISP794, a strain 8325 derivative that, like the multiply marked parent strains ISP933, ISP983, and ISP988, carries these three prophages.

Several auxotrophic markers were expressed only rarely among the recombinants and, as a result, never exhibited high CIFs with other

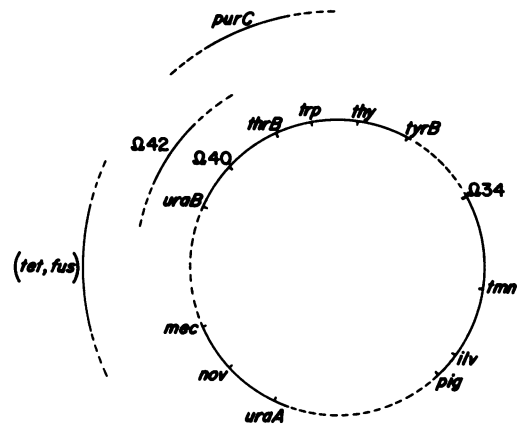

FIG. 2. Chromosome map of *S. aureus* NCTC 8325 defined by protoplast fusion. The orientation of the three major linkage groups and the approximate locations of the *purC193*::Tn*551*, Ω(Chr::Tn*551*)*42*, *tet-3490*, and *fus-149* markers are based on the results of protoplast fusion analysis. Markers joined by solid lines are known to be linked to one another by transformation (29).

markers known to be closely linked. The *ala-126* marker illustrates this situation. Recovery of Thy^-, His^-, Pur^-, or Pig^+ recombinants was also reduced. Analysis of the crossover patterns with these markers showed that the prototrophic alleles of these markers were introduced into recombinants as a consequence of crossovers on either side. In contrast, whereas the Tyr and Trp markers consistently had low CIFs, Tyr and Tmn often were coinherited at relatively high frequency (and are known to be linked by transformation [35]). One explanation for these findings may be that the region between Trp and Tyr includes either the origin or terminus of chromosome replication. Gabor and Hotchkiss (10) found that crossover activity near the origin and terminus of the *B. subtilis* chromosome was 20 times more frequent than in other regions of the chromosome. In contrast to the auxotrophic markers, the antibiotic resistance determinants used in this study were excellent for use in fusion analysis, coinheriting predictably with markers known to be linked to them.

The necessity of using isogenic parental strains for fusion analyses and the observation that some markers, such as *ala-126*, were not inherited solely as a function of their map location demand that caution be exercised in applying protoplast fusion analysis to genomic mapping in other microorganisms. A major contributing factor to the success of these studies in *S. aureus* was the availability of sets of markers whose linkage relationships were known from transformation analyses. This situation made it possible to evaluate the results of

fusion crosses and to recognize a marker that failed to appear among the recombinants according to its map position. Moreover, the availability of transformation allowed new linkages identified by protoplast fusion to be tested by transformation, which is free of the ambiguities inherent in fusion analysis (35). Without some means of confirming the linkages identified by fusion, it would be very difficult to identify the markers involved in fusion analysis that were responsible for spurious data because of anomalous inheritance among recombinants.

The early stages of this investigation were conducted without a microcomputer; under these circumstances, the scoring and analysis of data from 9- and 10-factor crosses was prohibitively time consuming and error prone. The use of a programmed microcomputer to sort the scoring data into phenotypes and determine the frequencies at which they occurred was essential in developing chromosome mapping by protoplast fusion. Although the most valuable and informative data were the CIFs, the ability to calculate the minimum number of crossovers required to account for different sequences of markers was also very useful. Also, because the scoring data from each experiment were stored on a magnetic disk, program modifications for the analysis and treatment of the data could be tested rapidly.

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