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CELL FUSION BETWEEN L-FORMS AND PROTOPLASTS OF *STAPHYLOCOCCUS AUREUS*

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SUMMARY Mixtures of various combinations of Lysostaphin protoplasts and stable L-forms of *Staphylococcus aureus*, which have different markers for drug resistance, were treated with polyethylene glycol (PEG) to examine the development of doubly resistant fusion products (fusants). To recover doubly resistant colonies as L-forms, they were incubated in 4.5% NaCl-brain heart infusion (BHI) broth containing penicillin G (PCG) for enrichment culture and cultured in PCG-4.5% NaCl-BHI agar medium (method 1), while to recover doubly resistant fusants as L-forms and coccal forms, they were grown on reversion medium (R medium) which causes reversion of protoplasts or fusants to parent type cells, and then cultured on assay media, i.e., R medium, BHI agar medium or PCG-4.5% NaCl-BHI agar medium (method 2).

Under both experimental conditions, doubly resistant fusants developed as L-form cells by PEG treatment of pairs of protoplasts carrying the chloramphenicol (CP)-resistance plasmid and L-forms having chromosomal resistance to streptomycin (SM). In the reverse combinations, i.e., protoplasts showing chromosomal SM-resistance and L-form cells carrying the CP-resistance plasmid, the first method gave no doubly resistant colonies. By the second method, without enrichment culture on R medium, the latter combination gave doubly resistant fusants as L-form, coccal-type and mixed-type colonial forms, while when the PEG-treated mixture was enriched on R medium, fusants were obtained exclusively as the coccal type on either R mdeium or BHI agar assay medium.

Neither of the methods yielded colonies of doubly resistant fusants on PEG-treatment of pairs of protoplasts and L-forms both of which were chromosomal, but with different drug resistances.

These results show that PEG-induced cell fusion between protoplasts and L-forms of *S. aureus*, unlike the fusion between protoplasts or between L-forms, resulted in transfer of the drug resistance controlled by the plasmid to the fusion products. The fusants obtained were L-forms in method 1, and coccal type in the method 2.

INTRODUCTION

Studies on cell fusion of bacteria require cells with an exposed cytoplasmic membrane, but without a cell wall that blocks fusion, i.e., protoplasts or L-form cells. However, the uses of both these types of cells in fusion experiments have inherent problems: protoplasts must be produced efficiently from parent cells having a cell wall, and since, with a few exceptions, protoplasts cannot multiply, fused protoplasts must be reverted to the original type of cells. The conditions for reversion have so far been investigated only in a limited number of bacterial species including *Bacillus subtilis* and *Streptomyces* (Landman, Ryter and Fréher, 1968; Okanishi, Suzuki and Umezawa, 1974). Hence, the optimal conditions for reversion must be determined for each species of bacteria used for fusion experiments. Another problem with gram-negative bacteria is that they have a more intricate cell wall architecture than gram-positive organisms. Thus while protoplasts with a completely exposed cytoplasmic membrane can be obtained by enzymatic removal of peptidoglycan or suppression of its biosynthesis in gram-positive bacteria, these treatments are not effective with gram-negative bacteria, since after these treatments an appreciable amount of the outer membrane composed of lipopolysaccharides and lipoprotein still remains and interferes with cell fusion. In fact, there are only two reports on cell fusion experiments using cells without peptidoglycan derived from gram-negative organisms (generally termed spheroplasts because of the remnant of the outer membranes) by Tsenin et al. (1978) who used *Escherichia coli* and Coetzee et al. (1979) who used *Providencia alcalifaciens*.

On the other hand, the stable L-forms have the advantage for cell fusion of not requiring reversion of fusion products (fusants) to the parent type cells with a cell wall, since they can survive and grow. In fact stable L-forms have been obtained from a number of bacterial species, including both gram-positive and gram-negative organisms. Nevertheless, the L-forms all require carefully controlled culture conditions, and the rich medium supplemented with horse serum and the other constituents generally used for cultivation of L-forms does not permit the use of nutritional requirements as a marker in selecting fusants of cells. More importantly, the stable L-forms lack most of their cell surface layers, the extent varying in different species, but their cytoplasmic membrane can protect their internal structure and support their growth as " naked " cells. Therefore, it is highly probable that the properties of the cytoplasmic membrane are considerably different from those of intact cells with a cell wall. This poses problems in cell fusion experiments aimed at analyzing the moieties of the cell surface layer lost and the properties of the cytoplasmic membrane.

Protoplasts have been used in bacterial cell fusion studies in other laboratories (Foder and Alföldi, 1976; Schaeffer, Gami and Hotchkiss, 1976; Götz, Ahrné and Lindberg, 1981; Hopwood, Wright and Bibb, 1977; Gasson, 1980; Jones, D., Jones, W. A. and Woods, 1985). Since we first reported in 1979 that L-forms of *Staphylococcus aureus* produced recombinants on cell fusion (Hirachi, Kurono and Kotani, 1979; 1980), we have demonstrated intraspecies cell fusion (Hirachi et al., 1982) and even intergenus cell fusion using the L-forms of *E. coli* and *Pseudomonus aeruginosa* (Kurono et al., 1983), and *Streptococcus faecalis* and *S. aureus* (Hirachi et al., 1985).

Because studies on cell fusion between protoplasts and L-forms should be useful, we conducted cell fusion experiments using *S. aureus*, in which fusions between L-forms and between protoplasts (Kato et al., 1985b) have been achieved. The present article is the first report on successful cell fusion between protoplasts and stable L-form cells.

MATERIALS AND METHODS

1. *S. aureus strains* (*Table 1*)

Coccal strains: MS353 pMS6 $(CM)^r$ [MS353 (pCp)] carrying the chloramphenicol (CP)-resistance plasmid (Inoue et al., 1970) was provided by Prof. H. Hashimoto (Department of Microbiology, School of Medicine, Gunma University). Streptomycin (SM)-resistant and erythromycin (EM)-resistant mutants, MS353 (Sm^r) and MS353 (Em^r), were obtained as follows; overnight cultures of the MS353 (pCp) strain in brain heart infusion (BHI) broth (Difco Laboratories, Mich, USA) were transferred to BHI agar plates, and the resultant colonies were each inoculated onto a BHI agar plate with chloramphenicol (5 μg/ml, Sigma Chemical Co., Mo, USA) for examination of resistance to CP. One of the colonies that had lost the CP-resistance plasmid was thus selected and subcultured (MS353 strain). Then this strain was seeded onto a BHI agar plate containing SM (2,000 μg/ml, Meiji Seika Co., Tokyo), and the resultant colony was isolated as an MS353 (Sm^r) strain. To obtain an MS353 (Em^r) strain, we seeded one of the colonies of the MS353 strain onto a BHI agar plate supplemented with EM (2 μg/ml, Sigma), cultured it in liquid medium and then inoculated it onto a BHI agar plate containing EM (15 μg/ml), and isolated EM-resistant colonies. Since both of these SM- and EM-resistant strains were produced by mutations, their resistance was thought to be controlled by a chromosomal gene.

L-form strains: EMT (Sm^r), a strain with chromosomal SM resistance, was isolated from the STA-EMT-1 strain (abbreviated as EMT-L; a gift from Prof. I. Tadokoro, Department of Bacteriology, Yokohama City University Medical School) (Eda, Matsuoka and Tadokoro, 1972), by the same procedures as that described in the proceding paragraph. The L-form, FP-L (pCp) carrying the CP-resistance plasmid was produced by the cell fusion method (see Results). This strain did not revert to the coccal type during repeated subcultures in penicillin G (PCG, Meiji)-free media.

The resistant coccal form and L-form organisms thus obtained were maintained by subculture on 4.5% NaCl-BHI agar plates containing the maximum concentration of each drug for resistance. For use in cell fusion, the coccal form or L-form strain in stock culture was cultured in 4.5% NaCl-BHI broth containing the drug to which the organism was resistant (1,500 μg/ml for SM and 15 μg/ml for EM and CP) at 37 C for 6 h.

2. *Protoplasts*

Protoplasts were prepared by the method of Makino (1983) with minor modification.. About 5 ml portions (10^{10} cells) of the MS353 (pCp), MS353 (Sm^r) and MS353 (Em^r) cultures were centrifuged, and the cells were suspended in 10 ml aliquots of 7.5% NaCl-25 mM Tris-HCl buffer solution (pH 7.4) supplemented with 25 μg/ml of Lysostaphin (from *Staphylococcus staphylolyticus*, Sigma). The mixture was incubated at 37C for 90 min. The number of residual coccal form cells in the protoplast suspension was negligible when

TABLE 1. *L-Form and coccal strains of S. aureus used in the present study*

Type	Strain	Minimum inhibitory conc[a] of			
		SM	EM	CP	PCG
L-form	EMT-L	<63	<0.063	<2	>2,000
	EMT (Sm^r)	>2,000	<0.063	<2	>2,000
	FP-L (pCp)[b]	<63	<0.063	>16	>2,000
Coccal form	MS353 (pCp)[c]	<250	<0.25	>16	<1
	MS353 (Sm^r)[d]	>2,000	<0.25	<4	<1
	MS353 (Em^r)[d]	<63	>16	<4	<1

[a] SM, streptomycin (μg/ml). EM, erythromycin (μg/ml). CP, chloramphenicol (μg/ml). PCG, penicillin G (U/ml).

[b] FP-L (pCp) is the fusant between EMT-L and MS353 (pCp) (see text).

[c] MS353 (pCp) which carries the chroramphenicol resistance plasmid.

[d] MS353 (Sm^r) and MS353 (Em^r) are SM-resistant and EM-resistant mutants, respectively.

examined on a BHI agar plate free from an osmotic stabilizer, indicating practically complete change of coccal forms to protoplasts.

3. *Protoplast lysates*

The suspension containing 5×10^9 protoplasts was centrifuged, and the precipitated protoplasts were suspended in 0.7 ml of sterilized water and submitted to occasional vigorous shaking at room temperature for one h to burst cells. Then, 0.3 ml of 22.5% (w/v) NaCl solution was added to bring the final saline concentration to 7.5% for use as a protoplast lysate.

4. *Media*

Reversion medium (R medium): The medium consisted of 2% BHI, 0.5 M sodium succinate, 0.02 M $MgCl_2$, 0.01% bovine serum albumin (BSA, Fraction V; Sigma) and 0.6% agar (Grade I, Wako Pure Chem. Industries, Osaka). This medium will be described in detail in a separate paper (Kato et al., 1985a).

Assay medium: In experiments on detection of L-form colonies alone following PEG treatment to induce cell fusion, 4.5% NaCl-BHI agar (0.8%) medium supplemented with PCG (100 U/ml) was used. For isolation of both L-form colonies and colonies of cells reverted to the coccal type, the following three types of assay media were used for PEG treated cells that had been cultured on R medium: (i) BHI agar medium for counting coccal type cells alone, (ii) 4.5% NaCl-BHI agar medium supplemented with PCG for counting L-form cells alone, and (iii) R medium for recovery of both coccal and L-form cells. L-Form and coccal form colonies on R medium were distinguishable under an inverted microscope. L-Form colonies had a so-called fried-egg appearance, coccal type cell colonies were round, homogeneous and smooth and mixed type colonies generally had a fried-egg appearance with homogeneous, and smooth areas of the coccal type in some parts.

SM and EM added to the assay media at concentrations of 1,000 μg/ml and 10 μg/ml, respectively. Assay media were supplemented with SM and CP of concentrations of 1,000 μg/ml and 10 μg/ml, respectively.

5. *Fusion of L-form cells and protoplasts*

A protoplast suspension (10^9 cells in 1 ml) was added to precipitated L-form cells (10^9 cells) and centrifuged at $8,000\times g$ for 15 min, at 10 C. Then 1–1.5 ml of polyethylene glycol 6,000 [PEG (6,000), Wako; at a concentration of 50% (w/v) in 4.5% NaCl solution] was added. The mixture was gently stirred for 1 to 2 min in Thermo-mixer (Thermonics Co., Tokyo) at room temperature. In some experiments 0.2 ml of a lysate of 10^9 protoplasts instead of protoplasts was added to the L-form cell precipitate (10^9 cells) and treated with PEG as described above.

The following two types of experimental conditions were used after PEG treatment: One was the type used in previous experiments on fusion of L-forms (Kurono et al., 1983). A 3–4 ml aliquot of 4.5% NaCl-BHI broth was gently mixed with the PEG-treated mixture. The mixture was then centrifuged, and the supernatant containing PEG was removed. The precipitate was transferred to a culture tube in 10 ml of 4.5% NaCl-BHI broth. Then, promptly, or after overnight enrichment culture in 4.5% NaCl-BHI broth supplemented with PCG (100 U/ml), the mixture was serially diluted 10-fold with 4.5% NaCl-BHI broth, and a 1 ml aliquot of the appropriate dilution was cultured in PCG-4.5% NaCl-BHI agar assay medium by the pour plate method at 37 C for 5 days.

The second type of conditions was that used by Kato et al. (1985b). A PEG-treated mixture of protoplasts and L-form cells was mixed with 10 ml of 7.5% NaCl-BHI broth and serially diluted 10-fold with the same broth. When enrichment culture was omitted, a 0.1 ml aliquot of the appropriate dilution was inoculated onto an agar plate of assay R medium and cultured at 37 C for 5 days. When enrichment culture was done, a 0.1 ml portion of a 10^2- to 10^4-fold dilution was inoculated onto the R medium agar plate (in a Petri dish, 8.6 cm in diameter) for incubation at 37 C for 5 days. The colonies that developed on 5–7 plates were wiped off with a cotton swab and serially diluted 10-fold with 7.5% NaCl-BHI broth. Aliquots (0.1 ml) of the dilutions were inoculated onto the three assay media mentioned above and cultured at 37 C for 2–5 days.

6. *Reagent*

DNase-I (from bovine pancreas, DN-CL), used in some experiments, was purchased from Sigma.

RESULTS

1. *Recovery as L-forms of PEG-induced fusants of L-forms and protoplasts*

Tables 2 to 4 show the results of cell fusion between protoplasts of the coccal type MS353 (pCp) carrying the CP-resistance plasmid and L-forms derived from the EMT (Sm^r) strain, an SM-resistant mutant. As shown in Table 2, PEG treatment of the mixture of the two types of naked cells, with or without enrichment culture in PCG-4.5% NaCl-BHI broth, induced colonies showing double resistance to CP and SM at high frequency. The frequency expressed as the ratio of colonies in the medium containing the drugs to those in the control basal medium was 5.2×10^{-5} without, and 7.3×10^{-4} with enrichment culture. When L-forms and protoplasts were treated separately with PEG, no doubly resistant colonies were detectable, indicating negligible mutation under the present experimental conditions. In this connection, it is noteworthy that independent PEG treatment of protoplasts from the MS353 (pCp) strain alone produced no colonies in the control medium either, regardless of enrichment culture. This result indicates that under the present experimental conditions, protoplasts cannot grow, and do not revert to the coccal type. When the mixture of L-forms and protoplasts was not treated with PEG, no doubly resistant colonies were detectable, irrespective of enrichment culture, indicating the necessity for PEG treatment of the mixture of protoplasts and L-forms.

Results using lysates instead of protoplasts are shown in Table 3. Regardless of enrichment culture, no doubly resistant colonies were isolated from these combinations, indicating the absence of transformation of EMT (Sm^r) by the CP-resistance plasmid of the MS353 (pCp) strain.

Table 4 shows the effect of addition of DNase-I at a concentration of 10 μg/ml at all stage of the procedures (PEG treatment, dilution of the mixture and enrichment culture) to determine whether DNase-I treatment influenced the frequency of emergence of doubly resistant colonies. The frequency of doubly resistant colonies was not affected by the addition of DNase, with or without enrichment culture, indicating the absence of transformation by the drug-resistance genes

TABLE 2. *PEG-induced cell fusion between protoplasts derived from a coccal type, MS353 (pCp), and L-forms, EMT (Sm^r)*

Combination		PEG	Enrichment culture[b] (h)	No. of colonies[a] developed in assay medium, PCG-4.5% NaCl-BHI agar with		
Protoplast	L-form			CP+SM[d]	No addition	Ratio[c]
MS353 (pCp)	EMT (Sm^r)	+	0	1.4×10^{3}	2.7×10^{7}	5.2×10^{-5}
MS353 (pCp)	—	+		<1	<1	—
—	EMT (Sm^r)	+		<1	5.6×10^{8}	$<1.8\times10^{-9}$
MS353 (pCp)	EMT (Sm^r)	−		<1	3.2×10^{8}	$<3.1\times10^{-9}$
MS353 (pCp)	EMT (Sm^r)	+	18	2.7×10^{5}	3.7×10^{8}	7.3×10^{-4}
MS353 (pCp)	—	+		<1	<1	—
—	EMT (Sm^r)	+		<1	1.6×10^{9}	$<6.3\times10^{-10}$
MS353 (pCp)	EMT (Sm^r)	−		1.8×10	3.2×10^{8}	5.6×10^{-8}

[a] Mean CFU in triplicate cultures (per ml).
[b] Enrichment cultre in 4.5% NaCl-BHI broth containing penicillin G (100 U/ml).
[c] Ratio of colonis in medium containing the respective drugs to those in control medium without drugs.
[d] CP, 10 μg/ml. SM, 1,000 μg/ml.

TABLE 3. *Absence of induction of doubly drug-resistant fusants by PEG treatment of a mixture of osmotic lysates of protoplasts derived from MS353(pCp) and stable L-froms, EMT(Sm^r)*

Combination	PEG	Enrichment culture[b] (h)	No. of colonies[a] developed in assay medium, PCG-4.5% NaCl-BHI agar with		
			CP+SM	No addition	Ratio[c]
MS353 (pCp) protoplasts ×EMT (Sm^r)	+	0	4.0×10^{2}	4.4×10^{6}	9.1×10^{-5}
	−		<2	1.0×10^{8}	$<2.0\times10^{-8}$
MS353 (pCp) lysate[d] ×EMT (Sm^r)	+		<2	2.1×10^{7}	$<9.5\times10^{-8}$
	−		<2	1.1×10^{8}	$<1.8\times10^{-8}$
MS353 (pCp) protoplasts ×EMT (Sm^r)	+	18	5.0×10^{4}	1.3×10^{8}	3.8×10^{-4}
	−		2.5×10	2.8×10^{8}	8.9×10^{-8}
MS353 (pCp) lysate[d] ×EMT (Sm^r)	+		<2	2.5×10^{8}	$<8.0\times10^{-9}$
	−		<2	7.5×10^{7}	$<2.7\times10^{-8}$

[a,b,c] See legend to Table 2.

[d] See text.

TABLE 4. *Effects of DNase treatment on development of doubly drug-resistat fusants of protolpasts derived from MS353(pCp) and L-froms, EMT(Sm^r)*

Combination		PEG	DNase[d]	Enrichment culture[b] (h)	No. of colonies[a] developed in assay medium, PCG-4.5% NaCl-BHI agar with		
Protoplasts	L-forms				CP+SM	No addition	Ratio[c]
MS353 (pCp) ×	EMT (Sm^r)	+	−	0	2.3×10^{3}	1.0×10^{7}	2.3×10^{-4}
		+	+		2.2×10^{3}	9.0×10^{6}	2.4×10^{-4}
		−	−		<1	1.8×10^{8}	$<5.6\times10^{-9}$
		−	+		<1	2.8×10^{8}	$<3.6\times10^{-9}$
MS353 (pCp) ×	EMT (Sm^r)	+	−	18	7.0×10^{3}	1.6×10^{9}	4.4×10^{-6}
		+	+		1.4×10^{3}	2.0×10^{8}	7.0×10^{-6}
		−	−		1.3×10	5.6×10^{8}	2.3×10^{-8}
		−	+		2	2.9×10^{9}	6.9×10^{-10}

[a,b,c] See legend to Table 2.

[d] DNase-I (10 μg/ml) was added to the reaction mixture for PEG-treatment, and the medium for enrichment culture and dilution.

derived from L-forms and protoplasts.

The above results together demonstrate that PEG treatment of a mixture of protoplasts carrying the CP-resistance plasmid and L-forms from an SM-resistant mutant induces the development of doubly resistant colonies as L-form cells as a result of cell fusion, and not by other mechanisms.

Next, we attempted to achieve cell fusion of opposite combinations, i.e., between protoplasts derived from a drug-resistant mutant and L-forms carrying the drug-resistance plasmid, the FP-L (pCp) strain. These L-forms were obtained as follows: First, protoplasts from the coccal type MS353 (pCp) carrying the CP-resistance plasmid and L-forms sensitive to SM, EM and CP (EMT-L strain; naturally resistant to PCG) were sub-

TABLE 5. *PEG-induced cell fusion between protoplasts derived from MS353(pCp) and stable L-forms, EMT-L*

Combination		PEG	Enrichment culture[b] (h)	No. of colonies[a] developed in assay medium, PCG-4.5% NaCl-BHI agar with		
Protoplasts	L-forms			CP	No addition	Ratio[c]
MS353 (pCp)	× EMT-L	+	0	2.0×10^{2}	2.0×10^{7}	1.0×10^{-5}
		−		<10[d]	6.1×10^{7}	$<1.6\times10^{-7}$
MS353 (pCp)	× EMT-L	+	18	4.8×10^{3}	5.0×10^{6}	9.6×10^{-4}
		−		2	4.4×10^{6}	4.5×10^{-7}

[a,b,c] See legend to Table 2.

[d] Numerous small colonies developed when undiluted reaction mixture was seeded (see text).

TABLE 6. *PEG-induced cell fusion between protoplasts derived from a coccal type with chromosomal drug resistance and L-forms with a resistance plasmid or chromosomal gene*

Combination		PEG	Enrichment culture[b] (h)	No. of colonies[a] developed in assay medium, PCG-4.5% NaCl-BHI agar with			
Protoplasts	L-forms			SM+CP	SM+EM	No addition	Ratio[c]
MS353 (pCp)	× EMT (Sm^r)	+	0	1.3×10^{3}	—	2.3×10^{7}	5.7×10^{-5}
		−		4	—	1.8×10^{8}	2.2×10^{-8}
MS353 (Sm^r)	× FP-L (pCp)[d]	+		<2	—	3.1×10^{6}	$<6.5\times10^{-7}$
		−		<2	—	1.2×10^{7}	$<1.7\times10^{-7}$
MS353 (Em^r)	× EMT (Sm^r)	+		—	<2	2.6×10^{6}	$<7.7\times10^{-7}$
		−		—	<2	1.6×10^{7}	$<1.3\times10^{-7}$
MS353 (pCp)	× EMT (Sm^r)	+	18	1.4×10^{5}	—	2.8×10^{8}	5.0×10^{-4}
		−		8.5	—	1.4×10^{8}	6.1×10^{-8}
MS353 (Sm^r)	× FP-L (pCp)[d]	+		<2	—	9.0×10^{7}	$<2.2\times10^{-8}$
		−		<2	—	1.5×10^{8}	$<1.3\times10^{-8}$
MS353 (Em^r)	× EMT (Sm^r)	+		—	<2	9.3×10^{7}	$<2.2\times10^{-8}$
		−		—	<2	1.8×10^{7}	$<1.1\times10^{-7}$

[a,b,c] See legend to Table 2.

[d] The fusants between MS353 (pCp) and EMT-L.

mitted to cell fusion, and one of the colonies (fusants) appearing on the PCG-4.5% NaCl-BHI agar plate supplemented with CP (10 μg/ml) and PCG (100 U/ml) was isolated and maintained in pure culture (Table 5).

Table 6 shows the results for the combination of protoplasts from the coccal type SM-resistant mutant, MS353 (Sm^r) and L-forms carrying the CP-resistance plasmid, [FP-L (pCp) strain], and for the combination of protoplasts and L-forms both having the drug-resistant chromosomal marker [protoplasts from the EM-resistant coccal type, MS353 (Em^r) and SM-resistant L-form, EMT (Sm^r)]. Under experimental conditions in which doubly resistant colonies developed at a high frequency from the control mixture of MS353 (pCp) and EMT (Sm^r), the former combinations produced no colonies doubly resistant to SM and CP, and the latter, no colonies

TABLE 7. *Recovery of PEG-induced fusants of protoplasts and L-forms by using R medium for assay (without enrichment culture)*

Combination		PEG	No. of colonies[a] developed on assay medium, R medium with		
Protoplasts	L-forms		SM+CP or SM+EM	No addition	Ratio[c]
MS353 (pCp) ×	EMT (Sm^r)	+	2.6×10^{3}[b]	1.7×10^{8}	1.5×10^{-5}
		−	<10	3.7×10^{7}	$<2.7\times10^{-7}$
MS353 (Sm^r) ×	FP-L (pCp)	+	7.1×10^{3}[d]	2.2×10^{7}	3.2×10^{-4}
		−	<10	4.1×10^{7}	$<2.4\times10^{-7}$
MS353 (Em^r) ×	EMT (Sm^r)	+	<10	3.4×10^{6}	$<2.9\times10^{-6}$
		−	<10	1.6×10^{7}	$<6.3\times10^{-7}$

[a],[c] See legend to Table 2.
[b] All colonies were L-form.
[d] Colonies were either L-form, coccal form or mixed type.

TABLE 8. *Recovery of PEG-induced fusants of protoplasts and L-forms by enrichment culture on R medium*

Combination		PEG	No. of colonies[a] (ratio[c]) developed					
			R medium with		PCG-4.5% NaCl-BHI agar with		BHi medium with	
Protoplasts	L-forms		SM+CP or SM+EM	No addition	SM+CP or SM+EM	No addition	SM+CP or SM+EM	No addition
MS353 (pCp) ×	EMT (Sm^r)	+	<10	2.6×10^{11}	<10	4.5×10^{5}	<10	$<3.2\times10^{11}$
		−	<10	1.1×10^{11}	<10	3.4×10^{5}	<10	$<2.5\times10^{11}$
MS353 (Sm^r) ×	FP-L (pCp)	+	7.6×10^{5}[b] (2.5×10^{-4})	3.1×10^{9}	<10	8.3×10^{4}	1.1×10^{6}[b] (2.3×10^{-4})	4.7×10^{9}
		−	<10	8.7×10^{9}	<10	5.0×10^{4}	<10	$<8.0\times10^{9}$
MS353 (Em^r) ×	EMT (Sm^r)	+	<10	2.5×10^{11}	<10	5.9×10^{8}	<10	$<1.8\times10^{11}$
		−	<10	2.8×10^{11}	<10	6.9×10^{8}	<10	$<1.9\times10^{11}$

[a],[c] See legend to Table 2.
[b] All colonies were coccal form.

doubly resistant to SM and EM. We could not obtain doubly resistant fusants as L-forms cells by fusion of protoplasts from the coccus having the chromosomal resistance gene and L-form strains showing resistance of either plasmid or chromosomal origin.

2. *Reversion of PEG-induced fusions between L-forms and protoplasts to the coccal type*

Tables 7 and 8 show the frequencies of development of doubly resistant bacteria from the three pairs of combinations shown in Table 6 when the PEG treated mixtures were directly inoculated onto assay R medium, or when they were cultured on R medium and then inoculated onto three types of assay media.

As shown in Table 7, on combination of protoplasts derived from the MS353 (pCp) strain carrying the CP-resistance plasmid and

L-forms [EMT (Sm^r)], the chromosomal SM-resistant strain, produced doubly resistant L-form colonies of fried-egg form at high frequency (1.5×10^{-5}) when the PEG-treated cell mixture was directly (without enrichment culture) seeded on R medium containing SM and CP. The colonies developing on the R medium did not revert to the coccal type at least during the 10 day period. When the combination of L-forms carrying the CP-resistance plasmid [FP-L (pCp)] and protoplasts derived from the SM-resistant strain [MS353 (Sm^r)] was directly seeded on R medium containing SM and CP, doubly resistant fusants in colonial forms of the coccal type, L-form and mixed type, were obtained with a total frequency of 3.2×10^{-4} of the three types, the proportion of the coccal type increasing with the time of culture.

Table 8 shows the frequencies of doubly resistant bacteria when the PEG-treated mixtures were incubated on R medium for enrichment culture. With combinations of protoplasts derived from the MS353 (pCp) strain carrying the CP-resistance plasmid and the L-form with the SM-resistance gene [EMT (Sm^r)], no doubly resistant colonies were detectable in any of the media (i.e., R medium, PCG-4.5% NaCl-BHI medium and BHI medium all supplemented with CP and SM). In contrast, coccal type doubly resistant colonies appeared at high frequencies (2.5×10^{-4} and 2.3×10^{-4}) with reverse combinations [protoplasts from MS353 (Sm^r) and L-forms FP-L (pCp)] in R medium and BHI medium. However, PCG-4.5% NaCl-BHI medium produced no colonies, including those of the fried-egg type. Furthermore, doubly resistant colonies were not obtained from a combination of protoplasts and L-forms both with chromosomal drug resistance.

It may be added here that when these three combinations were enriched on R medium, the number of L-form colonies recovered on PCG-4.5% NaCl-BHI medium was unexpectedly small, regardless of PEG treatment.

DISCUSSION

We previously reported that PEG treatment and subsequent enrichment culture of a mixture of *S. aureus* L-form cells having different drug-resistant markers of chromosomal nature produced recombinants showing double resistance to the drugs used as markers as a result of cell fusion between substrains of the species (Hirachi et al., 1979; 1980).

In studies on fusion of bacterial cells, protoplasts of gram-positive organisms have been used exclusively in other laboratories, whereas we have been using stable L-form cells. Protoplasts and L-forms have the merits and demerits described in the introduction as materials in cell fusion experiments. In the present study, we attempted to induce cell fusion between protoplasts and stable L-forms derived from *S. aureus*, aiming to reduce the demerits and increase the merits by combination of the two cell types.

With combinations of stable L-forms showing chromosomal SM resistance and protoplasts carrying the CP-resistance plasmid, all cells obtained by both method were doubly resistant to CP and SM and grew as L-form colonies, with no reversion to the coccal type despite continued culture on R medium. In other words, we failed to obtain doubly resistant fusants with the protoplast-associated trait of ability of reversion to the coccal type.

In the reverse combination of protoplasts showing chromosomal resistance to SM and L-forms carrying the CP-resistance plasmid, no doubly resistant fusants were obtained on culture in 4.5% NaCl-BHI agar medium containing PCG in addition to SM and CP, even with enrichment culture after PEG treatment. However, culture of the mixture on R medium containing SM and CP, without enrichment culture after PEG treatment, gave three types of doubly resistant colonies, L-form, coccal type and mixed type colonies.

Furthermore, doubly resistant colonies of the L-form and mixed type that appeared on

PCG-free R medium reverted to coccal type colonies with increase in the time of culture. When the mixture was enriched on R medium after PEG treatment, doubly resistant fusants all formed coccal type colonies. Therefore, the doubly resistant fusants derived from this combination inherited the protoplast-associated trait of reversion to the coccal type.

With the last combination in which both the protoplasts and L-form showed chromosomal drug resistance, neither method yielded doubly resistant recombinants. This was unexpected, for cell fusion between L-forms [EMT (Sm^r)×EMT (Em^r)] and between protoplasts [MS353 (Sm^r)×MS353 (Em^r)] produced recombinants doubly resistant to SM and EM in our previous studies (Hirachi et al., 1979; 1980; Kato et al., 1985b). This difference may have been because the coccal type MS353 strain from which protoplasts were derived and the L-form EMT-L strain used as partners in the present study had different origins. Therefore, we attempted to obtain protoplasts from the coccal type Tazaki strain, the parent cell of the EMT-L, instead of the MS353 (Em^r) strain, for cell fusion with the EMT (Sm^r). However, reversion of protoplasts of the Tazaki strain to the coccal type occurred on R medium at a very low frequency (below 10^{-5}), thus preventing us from obtaining results.

Materson et al. (1983), and Stahl and Pattee (1983) reported that in cell fusion between protoplasts of *S. aureus*, recombination of chromosomal genes required treatment of the protoplasts with $CaCl_2$ as well as PEG. However, we found that fusion between L-forms and L-forms or between protoplasts and protoplasts of *S. aureus* resulted in the development of recombinants without $CaCl_2$ treatment, and that even with $CaCl_2$ treatment no recombinants were detected between L-forms and protoplasts.

The finding that no recombinants developed from a combination of protoplasts and L-forms both showing chromosomal resistance seems consistent with the results obtained with combinations between one partner (either L-form or protoplast) carrying the CP-resistance plasmid and the other showing chromosomal resistance to SM, i.e., doubly resistant fusants with the trait of the partner showing chromosomal SM-resistance were obtained exclusively, and no fusants with the trait of the partner carrying the CP-resistant plasmid were recovered. Thus, only the plasmid may be transferred in cell fusion between protoplasts and L-forms. Nevertheless, there may be other mechanisms that prevent the development of recombinants between stable L-forms that lack a cell wall throughout long-term subculture and protoplasts from which the cell wall has been temporarily removed.

The present results indicate that the doubly resistant cells appearing when a mixture of protoplasts and L-forms was treated with PEG were the results of cell fusion. Involvement of conjugation may be excluded by the absence of report on successful conjugation of L-forms or protoplasts of *S. aureus*, although that of the coccal type has been reported (McDonnell, Sweeney and Cohen, 1983), and also by the finding that no bacteriophages infecting L-form cells and protoplasts have yet been discovered, though we have found that L-forms contain prophages (Takemasa et al., 1984).

When the PEG-treated mixture of protoplasts from the MS353 (pCp) strain and L-forms from the EMT-L strain was pour-plated in 4.5% NaCl-BHI agar medium supplemented with CP and PCG without dilution, numerous colonies developed, whereas when the PEG-treated mixture was diluted 10-fold with 4.5% NaCl-BHI broth before inoculation into the medium no colony formation was observed (Table 5). These phenomena may be explained as follows: Protoplasts from the MS353 (pCp) strain may be unable to grow in the presence of CP and PCG, but may produce a CP-inactivating enzyme that is controlled by the plasmid. This enzyme may be liberated from protoplasts to inactivate

CP in the assay medium, permitting the CP-sensitive L-forms (naturally resistant to PCG) to form colonies. When a PEG-treated mixture is diluted, the CP-inactivating enzyme derived from protoplasts may be too dilute to inactivate CP in the medium sufficiently, and so the residual CP may prevent L-forms from forming colonies.

When colonies obtained by enrichment culture on R medium, with or without PEG treatment, were seeded on PCG-4.5% NaCl-BHI agar plates, only a very few L-form colonies developed (Table 8). This suggests that coccal forms obtained by reversion of protoplasts on R medium grew more rapidly than did L-forms, suppressing colony formation by L-forms that require more controlled culture conditions than those for coccal forms. In addition, some L-forms growing on agar plate medium are known to invade the medium to form colonies, making it technically difficult to recover colonies.

REFERENCES

Coetzee, J. N., Sirgel, F. A., Leactsas, G. 1979. Genetic recombination in fused spheroplasts of *Providence alcalifaciens*. J. Gen. Microbiol. 114: 313–322.

Eda, T., Matusoka, S., Tadokoro, I. 1972. Studies on staphylococcal L-forms. II Growth and morphological characteristics in liquid medium. Jpn. J. Bacteriol. 27: 795–800. [In Japanese]

Foder, K., Alföldi, L. 1976. Fusion of protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. USA 73: 2147–2150.

Gasson, M. J. 1980. Production, regeneration and fusion of protoplasts in lactic streptococci. FEMS Microbiol. Letters 9: 99–102.

Göze, F., Ahrné, S., Lindberg, M. 1981. Plasmid transfer and genetic recombination by protoplast fusion in staphylococci. J. Bacteriol. 145: 74–81.

Hirachi, Y., Kato, Y., Matsumoto, T., Ueyama, S., Furuyama, S., Kurono, M., Toda, Y., Kotani, S. 1982. Isolation of recombinants doubly and triply drug-resistant to streptomycin, tetracycline and chloramphenicol by PEG-induced cell fusion of single resistant *Staphylococcus aureus* L-forms. Biken J. 25: 111–119.

Hirachi, Y., Kurono, M., Kotani, S. 1979. Polyethylene glycol-induced fusion of L-forms of *Staphylococcus aureus*. Biken J. 22: 25–29.

Hirachi, Y., Kurono, M., Kotani, S. 1980. Further evidence of polyethylene glycol-induced cell fusion of *Staphylococcus aureus* L-forms. Biken J. 23: 43–48.

Hirachi, Y., Toda, Y., Kato, Y., Kurono, M., Takemasa, N., Kotani, S. 1985. Intergenic cell fusion between L-form cells of *Streptococcus faecalis* and *Staphylococcus aureus*. *In* Recent Advances in Streptococci and Streptococcal Diseases. p 236–237. Kimura, Y., Kotani, S. and Shiokawa, Y. [ed] Reedbooks, Chertsey, Surrey.

Hopwood, D. A., Wright, H. M., Bibb, M. J. 1977. Genetic recombination through protoplast fusion in *streptomyces*. Nature 268: 171–174.

Inoue, M., Hashimoto, H., Yamagishi, S., Mitsuhashi, S. 1970. Transduction analysis of the genetic determinants for chloramphenicol resistance in staphylococci. Jpn. J. Microbiol. 14: 261–268.

Jones, D., Jones, W. A., Woods, D. R. 1985. Production of recombinations after protoplast fusion in *Clostridium acetobutylicum* P262. J. Gen. Microbiol. 131: 1213–1216.

Kato, Y., Hirachi, Y., Toda, Y., Takemasa, N., Kotani, S. 1985a. Effects of the composition of the reversion medium on changes to coccal forms and L-forms of *Staphylococcus aureus* Lysostaphin protoplasts. J. Osaka Univ. Dental Soc. 30: 296–303. [in Japanese with English Summary]

Kato, Y., Hirachi, Y., Toda, Y., Takemasa, N., Kotani, S. 1985b. Cell fusion between protoplasts and stable L-forms of *Staphylococcus aureus*; The 2nd report. Jpn. J. Bacteriol. 40: 375. [in Japanese]

Kurono, M., Hirachi, Y., Kato, Y., Toda, Y., Takemasa, N., Kotani, S., Takahashi, T., Tadokoro, I. 1983. Intergenus cell fusion between L-form cells of *Pseudomonas aeruginosa* and *Escherichia coli*. Biken J. 26: 103–111.

Landman, O. E., Ryter, A., Fréhel, C. 1968. Gelatin-induced reversion of protoplasts of *Bacillus*

subtilis to the bacillary form: Electron-microscopic and physical study. J. Bacteriol. 98: 2154–2170.

Makino, T. 1983. Induction of L-phase variant from protoplasts of *Staphylococcus aureus*. Microbiol. Immunol. 27: 749–755.

Masterson, R., David, W., Wiley, B., Rogolsky, M. 1983. Mutagenesis of extra-chromosomal genetic determinants for exfoliative toxin B and bacteriocin R_1 synthesis in *Staphylococcus aureus* after plasmid transfer by protoplast fusion. Infect. Immun. 42: 973–979.

McDonnell, R. W., Sweeney, H. M., Cohen, S. 1983. Conjugational transfer of gentamicin resistance plasmid intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 23: 151–160.

Okanishi, M., Suzuki, K., Umezawa, H. 1974. Formation and reversion of *Streptomycete* protoplasts: Cultural condition and morphological study. J. Gen. Microbiol. 80: 389–400.

Schaeffer, P., Gami, B., Hotchkiss, R. D. 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA 73: 2151–2155.

Stahl, M. L., Pattee, P. A. 1983. Computer-assisted chromosome mapping by protoplast fusion in *Staphylococcus aureus*. J. Bacteriol. 154: 394–405.

Takemasa, N., Hirachi, Y., Kato, Y., Kurono, M., Toda, Y., Kotani, S., Takahashi, T., Tadokoro, I., Shinagawa, K., Iida, Y. 1984. Demonstration of lysogeny in stable L-forms of *Staphylococcus aureus*. Biken J. 27: 177–181.

Tsenin, D. A., Karimove, G. A., Rybchin, V. N. 1978. Recombinations by fusion of protoplasts of *Escherichia coli* K 12. Dokl. Akad. Nauk SSSR 243: 1066–1068.