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SHORT COMMUNICATION

EFFECT OF THE COMPOSITION OF REVERSION MEDIUM ON CHANGE OF *STAPHYLOCOCCUS AUREUS* LYSOSTAPHYIN PROTOPLASTS TO COCCAL FORMS AND L-FORMS

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SUMMARY The experimental conditions under which protoplasts of *Staphylococcus aureus* strain MS353 (pCp) are converted to the coccal or L-form were investigated. Protoplasts prepared by treating coccal MS353 (pCp) strain with Lysostaphin formed various types of colonies (coccal form, L-form and mixed types) in about 50% yield when they were plated on reversion (R) medium consisting of 2% brain heart infusion, 0.5M sodium succinate, 0.01% bovine serum albumin, 20 mM $MgCl_2$ and 0.6% agar. The L-form type colonies with a typical fried-egg appearance that developed on the R medium at an early stage gradually reverted to the coccal form through a mixed type stage in which a high density area first appeared in the periphery of the colony and then spread throughout the colony. The use of modified R medium without $MgCl_2$ or R medium in which 0.5M sodium succinate as an osmotic stabilizer was replaced by 7.5% NaCl resulted in marked delay in the appearance of reverted cells. R medium without bovine serum albumin yielded atypical L-form type colonies, which contained masses of coccal cells with very irregular margins. On the other hand, R medium without $MgCl_2$ but with penicillin G supported devlopment of L-form type colonies at high rate (13–15%) from the inoculated protoplasts.

Cells without a cell wall have generally been used for fusion of bacteria, because a cell wall prevents close contact with the cytoplasmic membrane of partner cells: protoplasts obtained by enzymatic removal or by inhibition of biosythesis of cell wall peptidoglycan (Hopwood 1981; Götz, Ahrné and Lindberg, 1981) or stable L-forms that can survive and multiply without a cell wall (Hirachi, Kurono and Kotani, 1979, 1980; Hirachi et al., 1982, 1985; Kurono et al., 1983) have been used.

After protoplast fusion, the protoplasts resulting from fusion must revert to parent form cells with cell walls, since few proto-

TABLE 1. *Effects of omission of BSA and $MgCl_2$ from R medium and R-NaCl medium on colony formation of S. aureus strain MS353(pCp) without Lysostaphin treatment in the presence and absence of penicillin G*

Medium	Constituent omitted	Plate (PCG[b] not added)	Ratio of CFU[a] Expt 1	Expt 2	Plate (PCG added)	Expt 1	Expt 2
R	None	A	100	70	A′	0.047	0.021
	$MgCl_2$	B	1.0[c]	0.52[c]	B′	1.2	0.16
	BSA	C	115	79	C′	0.025	<0.000053
	$MgCl_2$ and BSA	D	0.19	ND[f]	D′	<0.000077	ND
R-NaCl	None	E	ND	84	E′	ND	0.0053
	$MgCl_2$	F	ND	89	F′	ND	0.018
	BSA	G	ND	84	G′	ND	<0.000053
	$MgCl_2$ and BSA	H	ND	89	H′	ND	0.003
BHI		I	100[d]	100[e]	I′	<0.0000077	ND

[a] Ratio (percent) of colony forming units (CFU) on the respective medium to that on BHI medium without peniciline G (plate I).
[b] Final concentration of penicillin G, 100 U/ml.
[c] All colonies were L-forms at an early stage of growth.
[d] 1.3×10^8/ml
[e] 1.9×10^7/ml
[f] Not determined.

plasts can grow to form colonies. Although a number of studies on protoplast formation in various bacterial species have been reported, only a few (Landman, Ryter and Frehel, 1968; Okanishi, Suzuki and Umezawa, 1974) have described conditions under which protoplasts revert efficiently to the parent form with a cell wall. Wrick and Rogers (1973) developed a reversion medium, DPA medium, that contains sodium succinate, $MgCl_2$ and bovine serum albumin as constituents, to obtain mass reversion of both protoplasts and unstable L-forms of *Bacillus subtilis* and *Bacillus licheniformis* to the bacillary phase. A reversion medium similar to that of Wrick and Rogers was used by Schaeffer, Gami and Hotchkiss (1976) for protoplast fusion. However, the roles of the various constituents of the reversion medium in growth and reversion of protoplasts and unstable L-form variants have not been studied in detail. Thus, we studied this problem.

S. aureus, strain MS353 pMS6(CM)^r described by Inoue et al. (1970) [MS353(pCp); a gift from Prof. H. Hashimoto, Department of Microbiology, School of Medicine, Gunma University] was cultured in brain heart infusion (BHI) broth (Difco Laboratories, Mich., USA) supplemented with 4.5% NaCl, at 37 C for 6 h. Volumes of 1–2 ml of the culture were centrifuged at $6,000\times g$ for 15 min and the precipitated cells were suspended in 5 ml of hypertonic buffer [25 mM Tris-HCl buffer (pH 7.5) supplemented with 7.5% NaCl as an osmotic stabilizer]. The cell suspension was mixed with 25 μg/ml of Lysostaphin (a staphylolytic enzyme derived from *Staphylococcus staphylolyticus*; Sigma Chemical Co., Mo., USA). After incubation at 37 C for 90 min, the reaction mixture was serially diluted 10-fold with BHI broth supplemented with 7.5% NaCl. Volumes of 0.1 ml of appropriate dilutions were inoculated onto the variously modified rever-

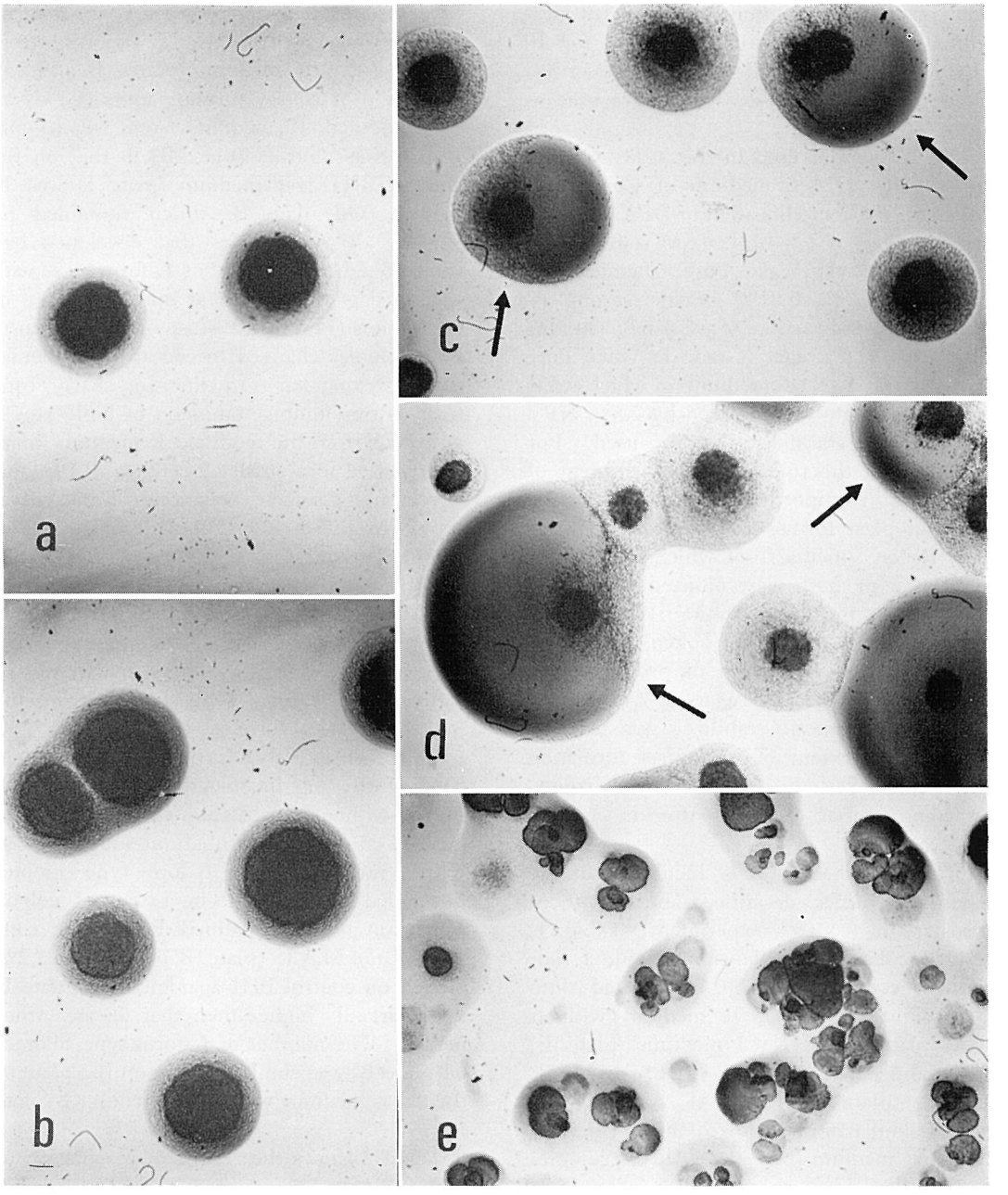

FIGURE 1. Photographs of L-form type and mixed type colonies
a. L-Form colonies developed on the modified R medium containing no $MgCl_2$ (plate B of Table 1) by inoculation of coccal form cells. ×40. b. L-Form type colonies which developed on the R medium supplemented with penicillin G and containing no $MgCl_2$ (plate B' of Table 1) by inoculation of coccal form cells. ×40. c and d. L-Form type and mixed type (indicated by arrows) colonies on the R medium (plate A of Table 2) by inoculation of Lysostaphin protoplasts. ×40. e. Mixed type colonies on the R medium containing no bovine serum albumin (plate C of Table 2) by inoculation of Lysostaphin protoplasts. ×40.

sion medium (R-medium) agar plates described below, and cultured at 37 C for 10 days. MS353(pCp) cells treated as described above but without Lysostaphin were used as controls.

The R medium used in this study was essentially the DM 3 medium of Chang and Cohen (1979), a modification of DPA medium, and consisted of 2% BHI, 0.5M sodium succinate (Grade I; Wako Pure Chemical Industries, Osaka), 0.01% bovine serum albumin (BSA, Fraction V; Sigma), 20 mM $MgCl_2$ and 0.6% agar (Grade I; Wako Pure Chemicals). R-NaCl medium, in which 0.5M sodium succinate was replaced by 7.5% NaCl as an osmotic stabilizer, was also used. For evaluation of the role of each constiutent of R medium in supporting the growth of coccal and protoplast forms of *S. aureus*, modified R or R-NaCl media from which either BSA or $MgCl_2$, or both were omitted were used. In addition, media containing 100 U/ml of penicillin G (Meiji Seika, Tokyo) were used to accelerate the reversion of protoplasts to L-forms. BHI (3.7%) agar (1%) medium without any osmotic stabilizer was used in checking the extent of protoplast formation on treatment with Lysostaphin and in counting colonies that appeared after inoculation of coccal cells.

First, coccal form cells incubated in the hypertonic buffer described above without Lysostaphin (control experiment, Table 1) were inoculated. As shown in Table 1 (left column) coccal form cells inoculated onto R medium (plate A), R medium without BSA (plate C), R-NaCl medium (plate E) and modified R-NaCl without BSA or $MgCl_2$ or both (plate F, G and H, respectively), formed similar numbers of colonies to that on BHI agar medium (plate I). All the colonies that developed on these media (pate A, C and F-I) showed the typical colonical morphology (round, homogeneous, smooth and turbid colony) of the *S. aureus* coccal form throughout the 10 day observation period. An unexpected finding was that the number of developing colonies was markedly reduced by omitting $MgCl_2$ or $MgCl_2$ and BSA from the modified R medium (plates B and D). The ratio of colony forming units (CFU) on the modified R medium from which only $MgCl_2$ was omitted (plate B) to that on the control BHI agar medium (plate I) was 1–0.52%. On plate B, which contained no $MgCl_2$, all the colonies that developed had the colonial morphology of L-forms, i.e. with a fried-egg appearance at an early stage of cultivation (Fig. 1a). Most of these L-form type colonies changed to coccal type colonies during expanded growth. In sharp contrast to this finding, omission of both $MgCl_2$ and BSA from the modified R medium (plate D) resulted in a small percentage (0.19%) of developing colonies exclusively of the coccal type. Modified R-NaCl medium (plates F or H) containing no $MgCl_2$ or neither $MgCl_2$ or BSA supported exclusive development of coccal type colonies in a similar way to control medium (plate I). This suggests that a high concentration of sodium succinate caused formation of L-form type colonies from the inoculated coccal form cells.

As shown in Table 1 (right column), when coccal form cells incubated in the hypertonic buffer without Lysostaphin were inoculated onto R or modified R medium supplemented with penicillin G, only L-form type colonies developed (Fig. 1b). The extent of colony formation on the modified R medium containing no $MgCl_2$ (plate B') was 0.16–1.2% of that on control BHI agar medium (plate I) and markedly higher than that on any other media. The number of L-form type colonies on plate B' was similar to that on the plate of the same medium without penicillin G (plate B).

Table 2 shows the results of inoculation of MS353(pCp) cells treated with Lysostaphin in the hypertonic reaction mixture onto the same type of R and modified R medium described in Table 1 (left column). Very few coccal form colonies were recovered on BHI agar medium without an osmotic stabilizer (plate

TABLE 2. *Effects of omission of BSA and $MgCl_2$ from R medium and R-NaCl medium on colony formation of Lysostaphin protoplasts of S. aureus strain MS353(pCp) in the presence and absence of penicillin G*

Medium	Constituent omitted	Ratio of CFU[a]					
		Plate (PCG[b] not added)	Expt 1	Expt 2	Plate (PCG added)	Expt 1	Expt 2
R	None	A	57	51	A′	1.1	0.78
	$MgCl_2$	B	49	38	B′	13	15
	BSA	C	12	18	C′	0.24	0.00053
	$MgCl_2$ and BSA	D	0.027	ND[c]	D′	<0.00077	ND
R-NaCl	None	E	ND	18	E′	ND	10
	$MgCl_2$	F	ND	13	F′	ND	6.8
	BSA	G	ND	12	G′	ND	7.9
	$MgCl_2$ and BSA	H	ND	2.9	H′	ND	0.041
BHI		I	0.00010	<0.0000053	I′	<0.0000077	ND

[a] Ratio (percent) of CFU of MS353 (pCp) protoplasts on the respective medium to that of coccal MS353 (pCp), from which the protoplasts were derived (see plate I in Table 1)

[b] See legend in Table 1.

[c] Not determined.

I). This indicates that the inoculum consisted almost exclusively of osmotically fragile protoplasts. The Lysostaphin protoplasts seeded on the R medium and the modified R medium containing no $MgCl_2$ (plate A and B, respectively) produced high percentage of colonies (38–57%). Omission of both $MgCl_2$ and BSA resulted in poor colony formation by protoplasts (0.027–2.9%), irrespective of the kind of osmotic stabilizer (sodium succinate or NaCl; plate D and H, respectively). On R medium (plate A) where a large number of colonies developed, the L-form type colonies that emerged after incubation for two days did not grow to large colonies or revert to coccal form colonies during further incubation. When an appropriate number of colonies had developed (300–100 colonies per plate), most of the colonies had the appearance of typical L-form type colonies at an early stage. However, during further incubation the number of these L-form type colonies decreased, and the number of coccal type colonies (homogeneous and smooth) gradually increased. In addition, mixed type colonies appeared as a transitional form between L-form and coccal type colonies. As shown in Fig 1c and 1d, these mixed type colonies showed an area of high density and development of coccal type colony within the fried-egg L-form type colony. The high density area spread over the L-form colonies until the reversion was complete. When plates had less than 100 colonies, L-form type colonies that had emerged on the R medium reverted to coccal type colonies at an early stage. Similar relationships of the number of developing colonies, the incubation time and the colony morphology were observed when various kinds of modified R medium were used for cultivation of protoplasts, except in modified R media that contained no BSA (plate C and D), where only atypical L-form type colonies with an irregularily-marginate coccal type area were produced from an early stage (Fig. 1e). Omission of $MgCl_2$ from the R medium (plate B) resulted in delayed appearance of

coccal type cells.

Modification of the R-NaCl medium by omission of $MgCl_2$ or BSA had no detectable effect on the reversion of protoplasts (plate F and G). Transition of colony morphology similar to that observed with modified R medium containing no $MgCl_2$ occurred during the period of cultivation on R-NaCl medium.

Protoplasts inoculated onto media supplemented with penicillin G produced only L-form type colonies, irrespective of the medium used (Table 2, right column). Modified R medium that did not contain $MgCl_2$ (plate B') yielded the largest number of colonies (more than ten times as many as on R medium, plate A'). Only a few colonies appeared on medium containing no BSA, or neither BSA or $MgCl_2$ but with penicillin G (plate C' and D'). On R-NaCl media supplemented with penicillin G, a high proportion of L-form type colonies developed (6.8–10%) except in R-NaCl medium that contained no BSA or $MgCl_2$ (plate H').

In conclusion, $MgCl_2$ and BSA, which are constituents of R medium, had strong influences on the rate of recovery, colony morphology and reversion process of inoculated coccal forms and protoplasts of *S. aureus*.

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