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Short Communication

Protoplast Transformation of *Staphylococcus carnosus* by Plasmid DNA

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Summary. Several coagulase-negative staphylococci were investigated for their ability to undergo polyethylene glycol-induced protoplast-transformation with plasmid DNA. Among this group of bacteria it was found that *S. carnosus* TM300 was superior in this respect. Several plasmids isolated from different species were readily transformed with an efficiency ranging between 4×10^6 to 2×10^3 transformants per µg plasmid DNA. Since *S. carnosus* is apathogenic and an important organism in food technology, this organism could be a suitable gram-positive strain for molecular cloning.

Certain micrococci have long been recognized as taking part in the fermentation of dry sausage (Niinivaara et al. 1956, 1957a and b). More recent studies have shown that many of these micrococci were wrongly classified and are in fact staphylococci (Fischer and Schleifer 1980). Isolates from dry sausage and from starter cultures used for the production of dry sausage were found to represent a new, coagulase-negative species of *Staphylococcus*, which was named *S. carnosus* (Schleifer and Fischer 1982). DNA-DNA hybridization studies indicated that strains of *S. carnosus* are closely related to one another, but share rather low DNA homology (10%–20%) with other staphylococci. A higher DNA homology (32%–39%) was found only with *S. simulans* ATCC 27848.

Since *S. carnosus* is apathogenic and an important organism in food technology, it is our aim to use this organism as a host strain for recombinant DNA and to study the genetic organization of certain staphylococci. One prerequisite for this is an effective transformation system. Plasmid transformation among the staphylococci has until now been restricted to the coagulase-positive species *S. aureus*, and was first described by Lindberg and Novick (1973) using competent *S. aureus* cells. The present communication describes the conditions for polyethylene glycol-induced plasmid DNA transformation of *S. carnosus* protoplasts.

Strains and Plasmids used are listed in Table 1. For plasmid isolation the organisms were cultivated in peptone-yeast extract broth containing 5% NaCl on an orbital shaker at 30° C for 24 h at which time the stationary phase was reached.

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Protoplast Formation and Protoplast Transformation was carried out as described previously (Chang and Cohen 1979; Götz et al. 1981) with the following modifications: A 20 ml overnight culture of *S. carnosus* (approximately 10^9 colony forming units per ml) was harvested and suspended in 20 ml SMMP medium. This medium was composed of: (a) 7.5 parts of SMM buffer (1 M sucrose, 0.04 M maleate, 0.04 M $MgCl_2$, pH 6.5); (b) 2 parts of 7% Penassay broth (Difco, USA); and (c) 0.5 parts of a 10% bovine serum albumin (BSA) solution. SMM buffer and Penassay broth were autoclaved separately; BSA was filter sterilized. Protoplasts were formed within 4 h at 37° C after addition of 10 µg lysostaphin and 20 mg lysozyme.

Protoplasts were harvested by centrifugation at $16{,}000 \times g$ for 30 min. The pellet was resuspended in 1 ml SMMP. Of the protoplast suspension 0.4 ml were mixed with 1 µg plasmid DNA and 2 ml 40% polyethylene glycol 6000 (PEG) in SMM buffer. After 2 min at room temperature 7 ml SMMP were added, gently mixed and then centrifuged at $16{,}000 \times g$ for 30 min. This sediment was resuspended in 1 ml SMMP and 0.1 ml samples of appropriate dilutions were plated onto DM3 medium which allowed cell wall regeneration (Wyrick and Rogers 1973; Chang and Cohen 1979). The protoplasts were incubated on this medium at 37° C for 3 h allowing phenotypic expression of the plasmid information.

The DM3 agar was overlaid with 3 ml soft agar containing the required antibiotics. This soft agar contained 10% tryptone (Oxoid), 10% yeast extract (Oxoid), 5% NaCl, 0.5% glucose, 0.05 M glycerol phosphate, 0.5 M Na-succinate (pH 7.3), 0.02 M $MgCl_2$, 0.05% BSA. Stock solutions of 1 M Na-succinate, 1.5 M glycerol phosphate, 50% glucose were separately autoclaved. At 50° C BSA and the required antibiotics were added. For selection of antibiotic-resistant clones erythromycin, 25 µg/ml; chloramphenicol, 10 µg/ml; tetracycline, 25 µg/ml; sodium arsenate, 4 mg/ml; kanamycin, 500 µg/ml were used.

The total protoplast regeneration rate and scoring of transformation frequency were calculated from colonies after incubation for 2–3 days on regeneration plates at 37° C.

S. carnosus is about 20 times more sensitive to lysostaphin than *S. aureus*. The optimal concentration of lysostaphin for *S. carnosus* was 0.5 µg lysostaphin per ml cell suspension in SMMP. Higher concentrations of lysostaphin led to an increased cell lysis concomitant with a reduction in the plasmid transformation frequency. The increased fra-

Table 1. Bacterial strains and plasmids

Species	Strain	Characteristics and derivation	Source or reference
Staphylococcus			
S. carnosus	TM300	wild type SK311	Schleifer and Fischer (1982)
S. aureus	RN11	strain 8325 carrying the penicillinase plasmid pI258	Novick (1967)
	SA113	restriction deficient mutant of strain 8325	Iordanescu and Surdeanu (1976)
	TM115	strain SA113 carrying the kanamycin resistance plasmid pUB110	Lacy and Chopra (1974)
	TM116	strain SA113 carrying the chlorampenicol resistance plasmid pC194	Iordanescu et al. (1978)
	TM117	strain SA113 carrying a small erythromycin resistance plasmid pE12, derived by in vivo recombination from pI258	this laboratory
	TM118	strain SA113 carrying a tetracycline and chloramphenicol resistance hybrid plasmid pCT20	(paper in preparation this laboratory)
S. simulans	MK148	wild type carrying a tetracycline resistance plasmid pMK148	Kloos and Schleifer (1975)
S. xylosus	DSM 20267	wild type carrying an arsenate, arsenite and antimony (III) resistance plasmid (pSX267)	Götz et al. (1983)
Bacillus			
B. subtilis	BR151	strain BR151 carrying a tetracycline resistance plasmid (pBC16) which was originally isolated from *B. cereus*	Kreft et al. (1978)

gility of *S. carnosus* cells was also reflected by the fact that both a higher sucrose concentration (0.75 M), and the presence of 0.5% BSA in the SMMP medium was necessary for optimal stabilization of protoplasts.

After PEG treatment *S. carnosus* protoplasts were incubated for at least 2 h on DM3 plates without antibiotics to enable phenotypic expression of genetic determinants carried by plasmids.

This phenotypic expression of *S. carnosus* protoplasts is also possible in the liquid DM3 medium containing 12.5% gelatine. In this case, the soft agar overlay is not required. SMMP or DM3 broth without gelatine is ineffective. This indicates that *S. carnosus* protoplasts need an agar or gelatine surface in order to be stable and biologically active.

The transformants were selected through the various resistance determinants expressed by the plasmid DNA (Table 2). The high concentration of succinate in the DM3 medium impairs the effectiveness of various antibacterial drugs like kanamycin and arsenate. Therefore, for selection of plasmid transformants a much higher concentration of these antibacterial drugs was necessary in DM3 plates than in peptone-yeast extract agar. For the selection of kanamycin and arsenate-resistant transformants, concentrations of 500 μg/ml, and 4 mg/ml, respectively, were necessary to avoid background growth of nontransformed cells. In the transformation system the protoplast concentration was normally of the order 2×10^8 per ml, determined by the regeneration frequency on DM3 plates.

The highest transformation frequency with 4×10^6 transformants per μg DNA was obtained with pC194. The transformation frequency of the other plasmids mentioned in Table 2 was in the range of 3×10^4–5×10^5 transformants per μg DNA, irrespective of the staphylococcal species from which the plasmids originated. A one order of magnitude lower transformation rate (2×10^3 transformants per μg of DNA) was obtained only with the tetracycline resistance plasmid pBC16, isolated from *Bacillus subtilis*.

Table 2. Transformation frequency of *S. carnosus* TM300 protoplasts by various plasmids

Plasmids	Isolated from	Size (kb)	Selection	Transformants μg DNA	Frequency of protoplast regeneration
pC194	*S. aureus*	2.9	Cm	4×10^6	5×10^8
pC194	*S. carnosus*	2.9	Cm	3×10^6	5×10^8
pMK148	*S. simulans*	4.5	Tc	3×10^4	2×10^8
pUB110	*S. aureus*	4.4	Km	6×10^4	4×10^8
pE12	*S. aureus*	2.5	Em	4×10^4	9×10^7
pCT20	*S. aureus*	5.3	Cm + Tc	3×10^4	2×10^8
pI258	*S. aureus*	28.2	Em	4×10^4	4×10^8
pSX267	*S. xylosus*	29.5	Asa	5×10^5	7×10^8
pBC16	*B. subtilis*	4.25	Tc	2×10^3	3×10^8

Cleared lysates were prepared as described by Novick and Bouanchaud (1971) and the plasmid DNA was concentrated with polyethylene glycol 6000 (Humphreys et al. 1975). ccc-DNA was isolated from CsCl-ethidium bromide gradients. To remove ethidium bromide the ccc-DNA was extracted with isopropanol (saturated with TE buffer; 10 mM Tris, pH 7.5 and 1 mM EDTA, which was saturated with CsCl), dialysed against TE buffer and precipitated with ethanol. Plasmid DNA and restricted DNA was subjected to agarose gel electrophoresis according to Sharp et al. (1973)

The frequency of transformation is obviously scarcely affected by the size of the plasmid, since the larger plasmids pI258 and pSX267 exhibited an equal or even higher transformation frequency than the smallest plasmid, pE12. There was also no difference in the transformation frequency when pC194 was isolated from *S. aureus* or from *S. carnosus*, indicating that the activity of the restriction modification system of *S. carnosus* protoplasts was markedly

reduced, as has already been found in protoplast fusion experiments with other staphylococci (Götz et al. 1981).

Since we have recently found that plasmid DNA was reorganized (unpublished results) in L-form fusion experiments between *S. warneri* and *S. aureus*, we also checked whether plasmids transformed into *S. carnosus* changed in size or restriction pattern. None of the plasmids isolated from *S. carnosus* was altered in size as compared to the plasmid isolated from the original strain (see Table 1). There was also no difference in the restriction patterns with *Eco*RI and *Mbo*I of the larger plasmids pI258, pSX267 or the recombinant plasmid pCT20.

Members of certain coagulase-negative staphylococcal species were investigated for their ability to undergo protoplast transformation with plasmid DNA. Among this group of bacteria it was found that *S. carnosus* TM300 was superior in this respect. This strain lacks the ability to produce protein A, coagulase, and hemolysin; criteria which are mainly associated with pathogenic *S. aureus* strains. On the other hand, the peptidoglycan is of the type L-Lys-Gly_{5-6} like *S. aureus* and renders *S. carnosus* highly susceptible to lysostaphin, a prerequisite for the preparation of protoplasts.

The introduction of a transformation system to *S. carnosus* TM300 suggests that this strain might be a suitable host for recombinant DNA derived from *S. aureus* or other organisms. The construction of plasmid vectors for this strain is underway.

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