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Temperature-Sensitive Mutants of *Staphylococcus aureus*: Isolation and Preliminary Characterization

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Abstract. Temperature-sensitive (ts) mutants of *Staphylococcus aureus* were isolated after mutagenesis with nitrosoguanidine and two cycles of enrichment with Penicillin G and D-Cycloserine. The mutants expressed tight, coasting, and leaky phenotypes on solid media. In broth, however, most exhibited coasting for a limited number of generations. The reversion frequency of selected ts mutants was less than 10^{-6}. Intraperitoneal (i.p.) immunization with ts mutant G/1/2 conferred significant protection (0 dead/6 total vs. 7/7, immunized vs. control; $p = 0.0006$) from lethal i.p. challenge with the parental wild-type (wt) *S. aureus* suspended in 5% porcine mucin, performed 28 days after i.p. administration of 10^8 colony-forming units. Protection induced by mutants of coasting phenotype was higher and lasted longer than that induced by mutants of the tight phenotype. The results of this study demonstrate that ts mutants of *S. aureus* can be obtained and that ts mutants are able to induce protective immunity from subsequent challenge with the parental wt strain.

Bovine mastitis is a disease of the mammary gland that significantly reduces milk production in dairy cows. Despite many efforts to control this disease during the past 20 years, it still inflicts significant economic losses on the dairy industry [3]. Gram-positive cocci, especially *Staphylococcus aureus*, have been singled out as the major cause of these losses. Immunization to prevent staphylococcal mastitis has been attempted before with different degrees of success [6, 13, 21]. Modern approaches to development of vaccines against bacterial pathogens aim at construction of live, attenuated strains that remain viable for a short period in the vaccinee without causing harm [7, 9, 14]. Temperature sensitivity as a method of genetic attenuation of bacteria has been suggested, and the feasibility of this approach to vaccine development has already been demonstrated with human [14] and ruminant [2] pathogens. This study was designed to obtain ts mutants of *S. aureus* and to establish whether immunization of mice with mutants of different ts phenotypes would confer protection from lethal challenge.

Materials and Methods

Bacteria and culture conditions. *S. aureus*, originally isolated from a cow with mastitis, was obtained from the Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina. Growth in Tryptic Soy broth (TSB) (Difco) occurred with mean generation times (MGT) of 25 min at 37°C and 47–49 min at 28°C, with aeration. Cultures in TSB were performed in nephelometric flasks unless otherwise indicated. A standard curve of absorbance vs. colony-forming units (cfu)/ml was obtained for routine estimation of the viable cells.

Isolation of ts mutants. Standard methods of chemical mutagenesis with nitroso-guanidine (Sigma Chemical Co., St. Louis, Missouri) and subsequent enrichment were used to isolate ts mutants of *S. aureus* [15]. Treatment with nitrosoguanidine induced 50% mortality and a 1300- to 1700-fold increase in mutation rate. The mutagen was removed by repeated centrifugation, and the culture was diluted and incubated at the permissive temperature (28°C) to allow segregation of the induced mutations. Log-phase cultures were shifted to the nonpermissive temperature (37°C), and, at various times thereafter, 10 units per ml Penicillin-G (Sigma) was added to enrich the cultures for ts mutants. Following a second cycle of enrichment with 30 mM D-Cycloserine (Sigma), the survivors were diluted appropriately, plated, and incubated at 28°C. Those colonies which appeared at 28°C were replica-plated with

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toothpicks to 37°C and 28°C, and the ts mutants were identified and isolated.

Characterization of ts mutants. The ts mutants of *S. aureus* were characterized with respect to phenotype on tryptic soy agar (TSA) and blood agar for initial screening, and in TSB for quantitation of residual replication at 37°C. Mutants of tight, coasting, and leaky phenotypes were isolated [15]. Mutants of tight phenotype cease all growth immediately after transfer to the nonpermissive temperature (37°C); those of coasting phenotype can undergo a limited number of divisions after transfer to 37°C, before ceasing growth; and those of leaky phenotype exhibit significantly impaired growth at 37°C, but are eventually able to form single colonies on solid media. Growth characteristics in liquid culture were determined spectrophotometrically and by viable counts. Reversion frequencies of ts mutants were estimated by incubating large numbers (10^8–10^9) of cells at 37°C. Coasting mutants were held at the nonpermissive temperature until they ceased all replication, before plating. Several biochemical characteristics of wt *S. aureus* and its ts derivatives were determined at 28°C, including production of coagulase, catalase, and deoxyribonuclease, and hemolysis on blood agar. To ascertain whether there were any major differences, besides temperature sensitivity, between the wt *S. aureus* and several ts derivatives, we determined the protein profiles by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Briefly, the organisms were cultured at 28°C in TSB enriched with 0.3 mg/ml glycine, and protoplasts were obtained by treatment with 30 μg/ml lysostaphin and 100 μg/ml lysozyme for 1 h. The enzymes and cell-wall proteins were removed by centrifugation, and protoplasts in the pellet were lysed by hypotonic shock in Tris buffer, pH 7.5. After treatment of the lysates with RNase and DNase, proteins were solubilized and subjected to SDS–PAGE according to a routine method [10]. In order to confirm the results obtained by SDS–PAGE of the proteins, small fragment restriction endonuclease analysis [8] was performed. Briefly, cells were cultured in glycine-enriched TSB, protoplasts were obtained by lysostaphin–lysozyme treatment, and chromosomal DNA was extracted according to a standard procedure [12]. Fingerprinting of *S. aureus* was performed after DNA digestion with endonuclease *Hin*dIII (New England Biolab, Inc., Beverly, Massachusetts) [19].

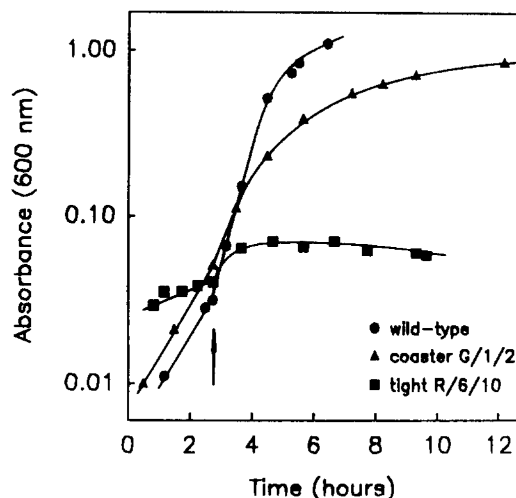

Fig. 1. Growth curves of the wild type, a tight and a coasting ts mutant of *S. aureus*. The arrow indicates the time at which the temperature was shifted from 28° to 37°C.

Animal studies. Preliminary immunological studies were performed with selected ts mutants to determine which phenotype induced the best protection from i.p. challenge. Groups of Swiss mice were immunized i.p. with 10^8 cfu suspended in 0.2 ml sterile saline. In some experiments, groups of mice received boosting i.p. injections with a similar dose 7 days later. All mice were challenged i.p. with 10 LD_{50} of a suspension of wt *S. aureus* in 5% porcine mucin. Morbidity and mortality were recorded, and the immunizing efficacies of the different phenotypes were compared statistically.

Results

In total, 43 ts mutants were obtained in three separate mutagenesis experiments with 20 μg/ml nitroso-

Table 1. Characteristics of selected ts mutants

Code	Phenotype on TSA	Reversion frequency	MGT (min) at 28°C	Replications at 37°C in TSB
C/3/15	Tight	1×10^{-4}	136	1
R/6/10	Tight	5×10^{-10}	130	1
R/11/15	Tight	4×10^{-6}	70	2
R/13/5	Tight	ND[a]	120	<1
A/1/53	Coasting	4×10^{-6}	80	2
C/3/20	Coasting	2×10^{-6}	50	3
F/2/19	Coasting	3×10^{-6}	74	3
G/1/1	Coasting	1×10^{-6}	55	4
G/1/2	Coasting	2×10^{-6}	74	5
J/2/3	Coasting	1×10^{-6}	50	4
P/1/17	Coasting	3×10^{-4}	65	2
P/7/11	Leaky	1×10^{-7}	60	NA[b]

[a] ND, not done.
[b] NA, not applicable; ts mutants of leaky phenotype continue replication until they reach stationary phase.

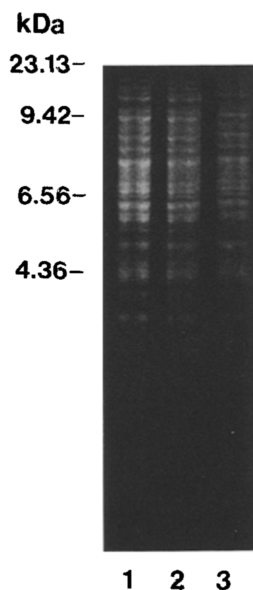

Fig. 2. Genomic fingerprinting of *S. aureus* ts mutants and the parental wt. The *Hin*dIII-digested DNA was subjected to electrophoresis in a 0.7% agarose gel applying 1 V/cm over a period of 14 h to the gel, which was stained afterwards with ethidium bromide. The lanes represent (1) the wt *S. aureus*, (2) ts mutant R/6/10, and (3) ts mutant G/1/2.

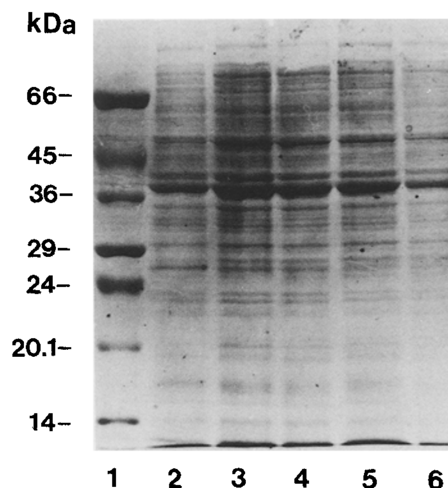

Fig. 3. SDS–PAGE of proteins from ts mutants, their revertants, and the parental wt *S. aureus*. Thirty micrograms of protein were loaded on each lane, and 15 V/cm was applied to the resolving gel. The lanes represent (1) molecular mass marker, (2) the wt *S. aureus*, (3) ts mutant R/6/10, (4) a revertant of R/6/10, (5) ts mutant G/1/2, and (6) a revertant of G/1/2. All bacteria were grown at 28°C.

guanidine. The ts mutant yield from the total number of cfu replica-plated varied from 3% to 4%. The growth curves of the wt *S. aureus* and two representative ts mutants, the tight R/6/10 and the coaster G/1/2, are shown in Fig. 1. Temperature sensitivity was maintained in broth, but in certain cases the phenotypes were not identical. Mutant R/6/10, for instance, had a tight phenotype on TSA but replicated once in TSB after the culture was shifted to the nonpermissive temperature. The reversion frequency of the ts mutants varied from 3×10^{-4} to 5×10^{-10} (Table 1). Fingerprinting analysis of ts mutants R/6/10 and G/1/2 and of the wt revealed no detectable differences in the *Hin*dIII DNA digestion patterns (Fig. 2). The SDS-PAGE protein profiles of these mutants were identical to those of the corresponding revertants and the wt (Fig. 3). Mutants R/6/10 and G/1/2 were selected for immunological characterization.

The highest level of protection induced by immunization with each of the three ts phenotypes was conferred by mutants of the coasting phenotype. The experiment was repeated three times, and the results from a representative one are shown in Table 2. Other experiments were designed to ascertain whether protection could be induced by a single i.p. administration of ts mutants R/6/10 (tight) and G/1/2 (coasting), and how long protection lasted. Immunization with coasting ts mutant G/1/2 induced significant protection that lasted at least 28 days, but mice injected with R/6/10 were not protected at all when challenged 14 days after i.p. immunization (Table 3).

Table 2. Protection induced by *S. aureus* ts mutants of different phenotype[a]

Mutant	Phenotype	Mortality (dead/total)	p[b]
R/6/10	tight	6/7	NS
G/1/2	coasting	0/7	0.001
A/1/53	coasting	1/7	0.005
P/7/11	leaky	3/7	NS
Control	—	5/7	

[a] Each mouse received 10^8 cfu of ts mutants by the i.p. route on days 0 and 7, and was challenged i.p. with 10 LD_{50} of wt *S. aureus* suspended in 5% porcine mucin on day 22.
[b] Level of significance when compared with the control group (Fisher's exact test). NS, not significant.

Discussion

Over the past 30 years several attempts have been made to immunize cows against staphylococcal mastitis. Most of these attempts, however, have met with little success, probably because of the use of

Table 3. Length of protection after immunization with *S. aureus* ts mutants R/6/10 and G/1/2[a]

Mutant	Immunization plan (CFU/mouse) day 0	day 7	day 14	day 21	Mortality (dead/total)	p[b]
R/6/10	—	—	—	10^8	1/6	0.0047
R/6/10	—	—	10^8	—	7/7	NS
R/6/10	—	10^8	—	—	8/8	NS
R/6/10	10^8	—	—	—	7/7	NS
G/1/2	—	—	—	10^8	0/6	0.0006
G/1/2	—	—	10^8	—	1/7	0.0023
G/1/2	—	10^8	—	—	1/8	0.0012
G/1/2	10^8	—	—	—	0/6	0.0006
Control	—	—	—	—	7/7	

[a] Mice were challenged by the i.p. route with 10 LD_{50} of wt *S. aureus* suspended in 5% porcine mucin, on day 28 of the experiment. The experiment was repeated four times under slightly different technical conditions and with different combinations of ts mutants in each experiment; the results were essentially the same.
[b] Level of significance when compared with the control group (Fisher's exact test). NS, not significant.

suboptimal doses, inappropriate vaccination routes, and inadequate regimens. Recently, a new vaccine composed of killed cells, a toxoid, and an adjuvant was reported [21]. The potential advantages of this vaccine for parenteral use and its mode of action have been recently summarized by Watson [20]. We hypothesize that a live, attenuated vaccine administered locally to the bovine mammary gland would induce protective immunity that would prevent colonization by *S. aureus*. Studies are currently under way in our laboratory to test the efficacy of *S. aureus* ts mutants in a murine model of mastitis.

Most animal isolates of *S. aureus* produce a thin polysaccharide capsule [17]. Yoshida et al. developed a capsular component vaccine for the prevention of bovine mastitis, but whether the protection induced was due to antibodies specific for the capsular polysaccharide of the immunizing strain was not demonstrated [22]. More recently, it was reported that immunization with purified capsular polysaccharide from *S. aureus* induced protection in experimental animals [11]. Other researchers have suggested that surface polysaccharides from *S. aureus* could serve as components of a subunit vaccine against straphylococcal disease not only in animals, but also in humans [5]. One question that remains to be answered is whether immunity against the *S. aureus* capsular polysaccharide is protective for cattle, because there are controversial results on the role of the *S. aureus* capsular polysaccharide in pathogenicity [1]. Combination of the *S. aureus* capsular polysaccharide with a toxoid carrier [4] is, in any event, an interesting approach that has had good results in other bacterial–host systems [18] and merits further investigation for *S. aureus*.

In conclusion, we isolated ts mutants of *S. aureus*, and we showed that immunization with these ts mutants induced protective immunity from subsequent challenge with the parental wt strain. Protection depended upon the phenotype of the ts mutants used for immunization, but the nature of the protective antigens involved remains to be elucidated. These results support the hypothesis that it should be possible to construct a live, attenuated vaccine composed of a mixture of strains expressing the most frequently isolated capsular polysaccharide serotypes [16]. In order to make the ts strains safe for veterinary application, at least two ts mutations would be combined in the chromosome to reduce the reversion frequency of the vaccine strains to acceptable levels.

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