Short Communications

In vivo Expression of Exoprotein Synthesis with a Sae Mutant of *Staphylococcus aureus*

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ABSTRACT

The expression of exoprotein synthesis of *Staphylococcus aureus* Sae mutant RC121 and its parental strain was studied under in vivo growth conditions. Cultures of both strains were inoculated into dialysis sacs implanted in sheep peritoneum. Results indicated that similar to in vitro grown mutant cells, Sae mutant RC121 shows diminished synthesis of α- and β-hemolysin, coagulase, DNase and protein A. However, in vitro and in vivo grown mutant cultures showed different exoprotein profiles in SDS-PAGE; some bands from in vivo mutant cultures were diminished or missing and others appeared as more concentrated, when compared with the pattern of the in vivo grown parental strain, while all the exoprotein bands from the in vitro cultures of the mutant were diminished or missing as compared to the in vitro grown parental strain. The virulence of the Sae mutant, assayed by intraperitoneal injection in mice, was lower than that of the parental strain after both in vivo and in vitro growth conditions.

RÉSUMÉ

L'expression de la synthèse des exoprotéines de la souche de *Staphylococcus aureus* (souche Sae) et du mutant RC121a été étudiée dans des conditions de culture in vivo en inoculant ces deux souches dans des sacs à dialyse implantés au niveau du péritoine de mouton. Les résultats indiquent que la culture in vivo du mutant RC121, tout comme sa culture in vitro, entraîne une diminution de la synthèse des hémolysines α et β, de la coagulase, de la désoxyribonucléase et de la protéine A. Des différences dans le profil électrophorétique des exoprotéines pouvaient être notées entre la culture in vitro et in vivo du mutant; lors de la comparaison entre le profil de la souche parentale et du mutant cultivés in vivo, certaines bandes du profil électrophorétique de la culture du mutant était diminuée d'intensité ou absente alors que d'autres apparaissaient plus intense comparativement à la souche parentale. Lors des cultures in vitro, toutes les bandes des exoprotéines du mutant étaient diminuées d'intensité ou absentes lorsque comparées à la souche parentale. La virulence du mutant Sae était moindre que celle de la souche parentale après culture dans des conditions in vivo et in vitro tel que démontrée par injection intrapéritonéale chez la souris.

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Staphylococcus aureus synthesizes numerous virulence determinants such as extracellular enzymes and toxins, cell-wall attached components and capsular material (1,2). Expression of some of these determinants has been shown to be controlled in the cell by global regulatory genes such as *agr*, *xpr* and *sar* (3,4,5). Expression of exoprotein production also varies in response to environmental signals (6,7). Thus, the different conditions that *S. aureus* as a pathogen encounters in a host may affect the expression of different virulence determinants. Several approaches have been undertaken to investigate aspects of the expression in vivo of several *S. aureus* characteristics. One of these approaches involved the comparison of *S. aureus* cells grown in dialysis sacs implanted in the sheep peritoneum with in vitro grown cells (8,9). The results of these studies showed that the in vivo grown cells expressed an additional cell-surface polysaccharide antigen as well as enhanced antiphagocytic properties. Growth of *S. aureus* inside an intraperitoneal device sealed with a 0.2 μm pore size filter was used by Arbuthnott et al (10) to analyze the expression of cell proteins and cell-bound protein A.

We have isolated and characterized transpositional mutants of *S. aureus*, designated Sae, that showed defective synthesis of α and β-hemolysins, coagulase, DNase and extracellular protein A (11,12). This work was aimed at the study of the expression of exoproteins and virulence of a Sae mutant in an in vivo system based on the growth inside a dialysis sac implanted in the sheep peritoneum, and its comparison with its parental strain and with in vitro growth conditions. Parental strain RC46, a wild-type *S. aureus* isolated from bovine mastitis (13), and strain RC121, a erythromycin-resistant Sae mutant derived from RC46 containing a *sae*::Tn917 mutation, have been described elsewhere (12).

For the in vivo experiments, overnight cultures of RC46 and RC121 grown in BHI broth and washed with PBS were inoculated with an initial concentration of 5×10^5 cfu/mL into dialysis sacs (molecular weight cut off 12000; Sigma Chemical Co., St. Louis, Missouri) containing 35 mL

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TABLE I. Extracellular proteins produced by RC46 and RC121 under in vivo and in vitro conditions

Enzymatic activity (U/mL)	In vivo						In vitro			
	8 h		24 h		48 h		6 h		24 h	
	RC46	RC121	RC46	RC121	RC46	RC121	RC46	RC121	RC46	RC121
α-hemolysin	—	—	2.3	—	12	1.2	10	—	100	5
β-hemolysin	128	—	119	7	1324	21	1600	60	9000	130
Coagulase	8	—	128	—	256	2	256	2	512	4
DNase ($\times 10^2$)	4	1	128	43	1024	85	1024	128	3200	1800

Values given are the mean of two measurements

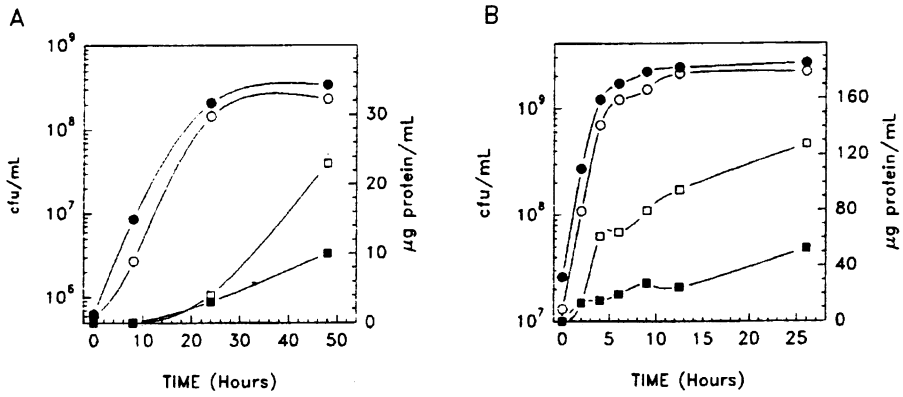

Figure 1. (A) Growth curves of (○) RC46 and (●) RC121 in dialysis sacs in the sheep peritoneum. (B) Same as (A) except that growth was carried out in BHI, at 37°C, with shaking. Concentration of extracellular proteins, under the corresponding growth conditions (A or B), was (□) for RC46, and (■) for RC121.

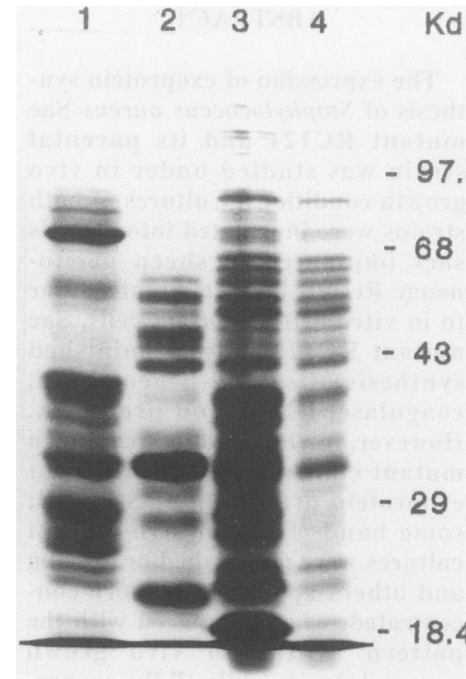

Figure 2. SDS-PAGE of extracellular proteins synthesized by parental and mutant strain under in vivo and in vitro growth conditions. Lanes 1 and 3: strain RC46; lanes 2 and 4: strain RC121. Concentrated proteins from 3 mL of 48 h in vivo (lanes 1 and 2) and 1 mL of 24 h in vitro (lanes 3 and 4) culture supernatant were separated on the gel and stained with Coomassie Blue R-250. Molecular markers are indicated on the right in kilodaltons (Kd).

of PBS. The sacs were implanted aseptically in the peritoneal cavity of the sheep as described by Watson and Prideaux (8). For the surgical procedure, sheep ($n = 8$) were sedated with xylaxine (0.2 mg/kg) and locally anesthetized with 15 mL of 2% lidocaine. Two dialysis sacs were introduced into each sheep and aseptically removed following the same surgical procedure at 8, 24 and 48 h after implantation. The content of the sacs were analyzed for cell numbers and extracellular protein content. The concentration of total extracellular proteins was determined from the culture supernatants by a modified Bradford method as described previously (11).

The two strains studied exhibited similar rates of growth, reaching a level of about 2×10^8 cfu/mL after 24 h of incubation (Fig. 1A). Both strains showed a post-exponential increase in exoprotein production; however, the maximal level of exoproteins produced by mutant RC121 was about two-fold lower than that of the parental strain. The decreased production of exoproteins by the Sae mutant in this in vivo system is similar to observations made after growth in vitro, in BHI broth (Fig. 1B), as well as with previous results reported for in vitro studies with this mutant and with a Sae mutant derived from another *S. aureus* strain (11,12). It can be seen that growth ceased in the in vivo system at a cell density of about 10-fold lower than that of the in vitro culture (10^8 vs 10^9 cfu/mL, respectively). This lowered growth might result from the limiting peritoneal nutrients able to pass through the 12000 cut off of the dialysis membrane and/or the less aerobic conditions.

The activities of different exoproteins synthesized by parental and mutant strains, determined under in vivo and in vitro conditions, are given in Table I. Enzymatic activities were quantified by serial dilutions of culture supernatant using 1% washed rabbit and sheep erythrocytes (14), rabbit plasma, and toluidine-DNA-agar (15) for α-hemolysin, β-hemolysin, coagulase and DNase determinations, respectively. In both growth conditions, the Sae mutant RC121 showed decreased or almost null production of α-hemolysin and coagulase and decreased production of β-hemolysin and DNase.

Extracellular proteins of supernatants from in vivo and in vitro grown cultures of the two strains were analyzed by 12.5% SDS-PAGE after precipitation by methanol-chloroform as described (16). Parental and mutant strains grown in vivo and in vitro gave different exoprotein profiles in SDS-PAGE (Fig. 2). As expected, the protein profile of the in vitro culture of the mutant showed a diminished concentration in most bands in comparison to that of in vitro grown parental strain (Fig. 2, lanes 4 vs 3). However, in the sample of the mutant culture grown in vivo, several proteins were decreased or missing while others appeared to be selectively expressed

compared to those of the in vivo grown parental culture (Fig. 2, lanes 2 vs 1).

Extracellular and cell-bound protein A synthesis was analyzed by Western immunoblot. Precipitated proteins from culture supernatant and cell-bound proteins released by lysostaphin treatment (17) were fractionated by 12.5% SDS-PAGE and electroblotted to nitrocellulose membranes. For antibody detection, the membranes were processed with rabbit antiserum prepared against commercial protein A (Sigma Chemical Co.) and horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co.) as primary and secondary antibodies, respectively. The conjugated antibody was detected using 4-chloro-1-naphthol. Under the in vivo conditions, only the 8 h sample of the parental strain, corresponding to the exponential growth phase, showed a band of protein A; there was no detectable protein A in the samples of the mutant culture (Fig. 3A). Analysis of extracellular protein A of supernatant of cultures grown in BHI broth in the exponential and post-exponential phases, revealed a marked decrease in the production of this protein by mutant RC121 (Fig. 3A). Cell-bound protein A was detected in the samples of in vivo cultures of the parental strain. The Sae mutant showed diminished levels of this protein. Similar results were obtained with the in vitro grown cultures (Fig. 3B). These observations on the presence of cell-bound protein A for in vivo cultures do not agree with the report by Arbuthnott et al (10) on the absence of the synthesis of this protein in the chamber implanted method employed in their study. These differences might be due to different methodology, different hosts and/or the different strains involved in each study.

For assays of virulence, samples of the in vivo cultures of the two strains under analysis recovered from the dialysis sacs 24 and 48 h after implantation, and 24 h samples of in vitro grown cells, were centrifuged and resuspended in sterile peptonated water. Groups of 10 Balb-c mice, 21–25 d old, were injected intraperitoneally with 1 mL of different bacterial concentrations (Table II). Mortality data, indicated in Table II, showed that mutant RC121 was less virulent than parental strain RC46 after in vivo and in vitro growth conditions. Data in Table II also indicate that wild type strain grown in vivo showed a higher virulence than when grown under in vitro conditions. This is in agreement with results reported by Watson (9) for a *S. aureus* strain inoculated in a similarly implanted dialysis sac.

TABLE II. Virulence in mice of RC46 and RC121 after in vivo or in vitro growth

	In vivo				In vitro	
	24 h		48 h		24 h	
(cfu/mL)	RC46	RC121	RC46	RC121	RC46	RC121
1.0×10^9	10	8	10	8	10	10
2.0×10^8	7*	1*	7	4	6	2
3.0×10^7	2	0	4*	0*	0	0
1.0×10^7	0	0	0	0	0	0

Data represent the number of dead mice in each group of 10 mice, registered 48 h post-inoculation
(*) RC46 is significantly different from RC121, $P = 0.01$ (Fisher's exact test)

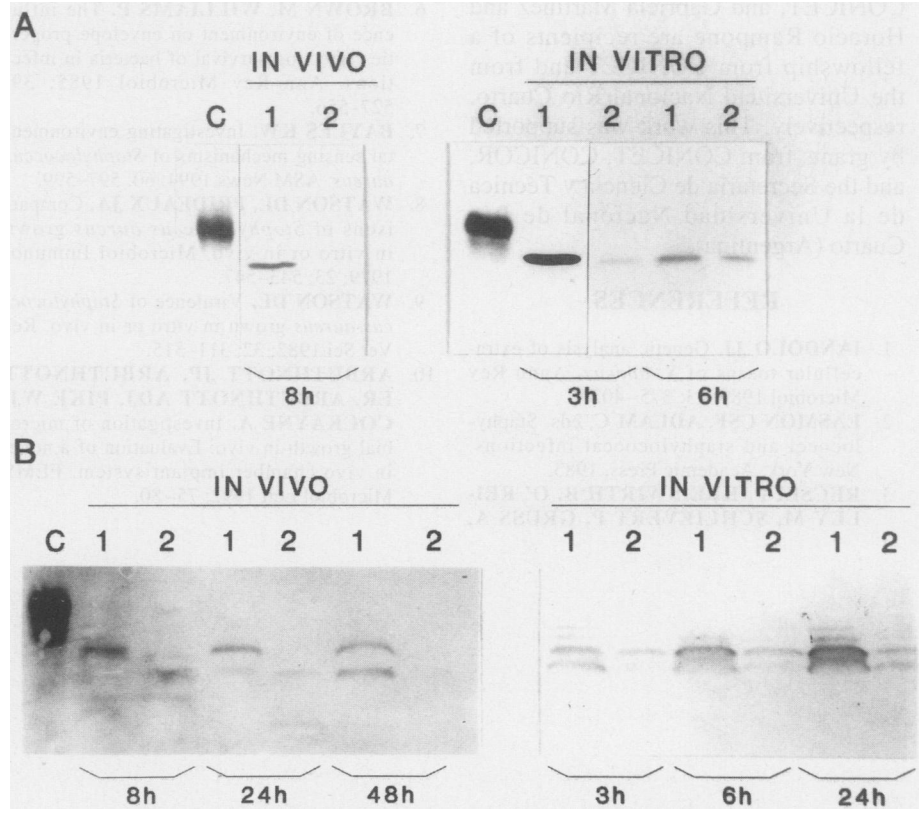

Figure 3. Western immunoblot analysis of (A) extracellular and (B) cell-bound protein A. Lanes 1: strain RC46; lanes 2: strain RC121; lanes C: commercial protein A. Concentrated extracellular proteins from 3 mL of in vivo and 1 mL of in vitro culture supernatants, and cell-bound proteins from 2.5 mg of cellular wet weight were applied to the gels.

The system used in this work allows the analysis of the production of exoproteins in vivo; it differs in this connection with the method employed by Arbuthnott et al (10), which only permits the analysis of cell proteins, due to the fact that the pore size of the membrane of the implanted chamber allows the passage of host proteins.

The data from this study indicate that the deficiency in the activities of α- and β-hemolysins, DNase, coagulase and protein A shown by the Sae mutant in vitro assays is also manifested with the in vivo procedure used. Accordingly, SDS-PAGE protein profiles from in vitro grown cultures of the Sae mutant reveal decreased protein expression; however, the protein bands of the Sae mutant from in vivo grown cultures shows diminished concentration of some bands and increased concentration of others as compared with the in vivo grown parental strain. This problem is being currently investigated. The Sae mutant is less virulent

than its parental strain in both the in vivo and in vitro systems.

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