Respiratory activity is essential for post-exponential-phase production of type 5 capsular polysaccharide by Staphylococcus aureus.

B Dassy and J M Fournier
Infect. Immun. **1996, 64(7):2408.**

INFECTION AND IMMUNITY, July 1996, p. 2408–2414 Vol. 64, No. 7
0019-9567/96/$04.00+0

Respiratory Activity Is Essential for Post-Exponential-Phase Production of Type 5 Capsular Polysaccharide by *Staphylococcus aureus*

BRUNO DASSY[1,2]* AND JEAN-MICHEL FOURNIER[1]

Département d'Ecologie, Institut Pasteur, F-75724 Paris Cedex 15,[1] and UFR des Sciences de la Vie, Division de Biochimie, Université Pierre et Marie Curie, F-75252 Paris Cedex 05,[2] France

Received 1 February 1996/Returned for modification 8 March 1996/Accepted 11 April 1996

Capsule formation is believed to have a significant role in bacterial virulence. To examine the possible involvement of capsular polysaccharide (CP) from *Staphylococcus aureus* in the pathological mechanisms associated with staphylococcal infections, we investigated the influence of respiratory activity on type 5 CP production by *S. aureus* grown in the presence of various concentrations of dissolved oxygen or nitrate. The effects of several metabolic inhibitors (arsenite, cyanide, azide, trimethylamine *N*-oxide, 2-heptyl-4-hydroxyquinoline *N*-oxide, and 2,4-dinitrophenol) were also tested. The metabolism of the bacteria was estimated by measuring their reductive capacity and by monitoring the pH and concentrations of fermentation products. Type 5 CP was always produced by *S. aureus* during the exponential phase of growth under all culture conditions tested. In contrast, post-exponential-phase CP production appeared to be strictly dependent on the respiratory activity. Since post-exponential-phase CP production contributes at least two-thirds of the total CP obtained, the influence of *S. aureus* respiration on CP production might be of some importance in the process of infection.

Staphylococcus aureus is responsible for numerous and various types of diseases, including septicemia, conjunctivitis, pneumonia, osteomyelitis, and endocarditis (42). Other bacterial species associated with invasive diseases produce a large capsule, and the role of capsule formation in disease has been extensively investigated with these species (11). Most *S. aureus* isolates do not produce a copious capsule, and it was not until recently that there emerged conclusive evidence that capsule formation by *S. aureus* is common among clinical isolates (1, 6, 9, 16). For most *S. aureus* strains, capsule formation is not visible under India ink but meets the definition of microcapsule (58). The capsular polysaccharides (CP) of *S. aureus* have been antigenically classified into at least eight different types (25). Two types of CP, types 5 and 8, account for between 60 and 90% of all clinical isolates (1, 6, 22). There is also evidence that these two types are the most common among *S. aureus* carried by healthy humans (1) and farm animals (39, 48).

In vivo, pathogenic bacteria encounter a variety of conditions not present in vitro and exhibit a wide array of adaptative regulatory responses. Oxygen tension varies between different sites in the host (36). Thus, at some stages of infection and disease, *S. aureus* may grow anaerobically (10), either by fermentation metabolism or by using nitrate as a terminal electron acceptor (7, 32, 45). Nitrate is readily available in the human host as an oxidation product derived from nitric oxide, a secretory messenger molecule synthesized by many mammalian cells (23). Investigations of the physiology of type 5 CP biosynthesis by *S. aureus* showed that oxygen availability enhanced CP production (14, 22, 39) and that the rate of CP production relative to the cell mass increased during the post-exponential phase of growth in batch culture (13, 14, 39). We report the influence of respiratory activity on type 5 CP production by *S. aureus* grown in the presence of various concentrations of dissolved oxygen or nitrate. The effects of several metabolic inhibitors were also tested. The metabolism of the bacteria was monitored by determining pH and fermentation product concentrations and by measuring their reductive capacity. The production of an exoprotein, acid phosphatase, was also determined as a control.

Under aerobic conditions, the main product of carbohydrate oxidation by *S. aureus* is acetic acid (18, 35, 45). This causes a decrease in the pH of the culture medium during exponential- and post-exponential-phase growth. After exhaustion of exogenous carbohydrate, acetate is reutilized (18, 40), and the bacteria can also use amino acids as energy sources via the tricarboxylic acid cycle, producing ammonia, which increases the pH during the stationary phase of growth (35). Under oxygen-limited conditions or reduced respiratory activity, the tricarboxylic acid cycle is weakly active and fermentation products, in particular acetoin, are secreted (45). Thus, acidification increases during growth and ammonia production is low during the stationary phase. Under completely anaerobic conditions, a variety of fermentation products, in particular lactate, are secreted and there is no ammonia production, leading to maximum acidification (18, 32).

Tetrazolium cations have long been utilized to measure cellular reductive capacity (5, 52, 54). These compounds accept electrons from oxidized substrates or appropriate coenzymes, thereby reducing them to a colored formazan product. The reduction of neotetrazolium (NT) by some bacteria, including *S. aureus*, is low under aerobic conditions and elevated under anaerobic conditions (5, 52). Reduction of tetrazolium cations by living cells correlates with the cellular NADH concentration (55), and therefore NT reduction to NT-diformazan (NTF) by *S. aureus* is a marker for the cellular NADH/NAD^+ ratio.

* Corresponding author. Mailing address: Unité du Choléra et des Vibrions, Département d'Ecologie, Institut Pasteur, 28 rue du Docteur Roux, F-75724 Paris Cedex 15, France. Phone: (33) 1 45.68.84.36. Fax: (33) 1 45.68.82.23. Electronic mail address: bdassy@pasteur.fr.

MATERIALS AND METHODS

Bacterial strain. *S. aureus* Reynolds is the prototype strain for type 5 CP (14, 25). It was stored lyophilized or in deep agar slants.

Culture medium. The modified Frantz medium (MFM-YE) described previ-

ously (14) was used. It is a semisynthetic medium containing mineral salts, lactose, glutamic acid, and yeast extract (Difco Laboratories, Detroit, Mich.) diffusate.

Growth conditions. Bacteria were grown in batch cultures at 37°C either in flasks or in a 6-liter fermentor. Flasks were inoculated with an overnight culture and placed in a rotary water bath shaker at 250 rpm. Unless otherwise specified, the medium did not exceed 15% of the flask volume. The working volume of the fermentor was 4.5 liters, and it was equipped with a draft tube agitation system (Biolaffite, Saint-Germain-en-Laye, France). An autoclavable, galvanic-type electrode was used to measure the dissolved oxygen tension. The pH was automatically controlled by the addition of 1 M NaOH or 1 M acetic acid. The culture was agitated at a constant speed of 500 rpm under nitrogen gas supplied at a rate of 1 liter/min for anaerobic culture. The fermentor was inoculated with 100 ml of an overnight culture in a shake flask.

Samples containing cells suspended in the growth medium were taken for analysis. Bacterial growth was monitored by measuring the optical density of an appropriate dilution of the cell suspension at 620 nm (OD_{620}). For 1 OD_{620} unit, plate counting of viable organisms gave a mean value of 5×10^9 bacteria per ml and total cell protein assays (38) gave a mean value of 75 μg/ml.

Aeration efficiency. The aeration efficiency of shake flasks filled with different volumes of the culture was determined by the sulfite oxidation method (17). Values are presented as the oxygen absorption rate (OAR) in millimoles of oxygen per liter of the culture per minute.

Type 5 CP assay. Bacterial cell suspensions were autoclaved at 121°C for 30 min to release type 5 CP from the cells (14). After centrifugation, the supernatant was retained and stored at −20°C prior to type 5 CP assay. This supernatant contained total CP (cell-bound CP plus culture fluid CP). The type 5 CP content was measured by a two-step inhibition enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies as previously described (6, 14) and is expressed in micrograms per milliliter.

Acid phosphatase. Phosphatase activity in the culture supernatant was determined according to the method of Barnes and Morris (3), with some modifications. The substrate, *p*-nitrophenylphosphate (Sigma Chemical, St. Louis, Mo.), was dissolved to a concentration of 1 mg/ml in 0.15 M acetate buffer, pH 5.5, containing 0.01 M $MgSO_4$. A 0.1-ml sample was added to 1 ml of the substrate, and the reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 2 ml of 0.075 M NaOH, and the yellow color was measured at 400 nm. The spectrophotometer was adjusted to zero with a blank to which 0.1 ml of noninoculated medium was added instead of sample. One unit of phosphatase activity is defined as the amount of enzyme which liberated 1 μmol of *p*-nitrophenol in 1 h.

NT reduction test. As described by Urban and Jarstrand (54), 5×10^9 bacteria were resuspended in 1 ml of phosphate-buffered saline, pH 7, containing 1 g of NT (Sigma) per liter and allowed to react in a glass tube at 37°C for 1 h. The tube was then centrifuged, and the pellet was extracted with 1 ml of dimethyl sulfoxide by vigorous shaking. The amount of NTF in the extract was measured by determination of the A_{570}. The blank consisted of bacteria resuspended in 1 ml of phosphate-buffered saline without NT. Absorbances were converted to concentrations by using a standard curve of NTF (ICN Biochemicals, Costa Mesa, Calif.) in dimethyl sulfoxide.

A washed cell suspension from the same sample was heated to 100°C for 15 min in 0.5 N NaOH containing 40 g of sodium dodecyl sulfate per liter to release cellular protein, which was determined by the method of Lowry et al. as modified by Peterson (38). Bovine serum albumin (Sigma) in the same conditions was used as the standard. NT reduction is expressed as milligrams of NTF per gram of cell protein.

Nitrite assay. Nitrite formed in the culture supernatant by bacterial nitrate reductase activity was determined by the routine diazotation reaction (33) adapted for a microtitration plate. To 130 μl of an appropriate dilution of the sample, 50 μl of 10-g/liter sulfanilic acid in 3 N HCl and 50 μl of 0.2-g/liter *N*-(1-naphthyl)ethylenediamine dihydrochloride were added. The plate was allowed to stand for 10 min at room temperature for color development, and the A_{570} was read. The blank was made with noninoculated medium, and a standard curve ranging from 3 to 30 μM nitrite was prepared.

Acetoin assay. Acetoin was assayed in the culture supernatant by the color reaction originally discovered by Voges and Proskauer (56) adapted for a microtitration plate. To 150 μl of the sample, 30 μl of 5-g/liter arginine and 30 μl of 50-g/liter α-naphthol in 2.5 N NaOH were added. The plate was allowed to stand for 1 h at room temperature for color development, and the A_{570} was read. The blank consisted of noninoculated medium, and a standard curve from 5 to 500 μM acetoin was prepared.

D-Lactic acid assay. The culture supernatant was assayed by the enzymatic procedure of Smith et al. (44) using D-lactate dehydrogenase (EC 1.1.1.28; Sigma). The blank was noninoculated medium, and samples containing known amounts of D-lactate were also tested to produce a standard curve.

Chemicals. Trimethylamine *N*-oxide (TMAO), 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), and 2,4-dinitrophenol were obtained from Sigma and dissolved in ethanol. All other chemicals were analytical or reagent grade and were purchased from Prolabo (Fontenay-sous-bois, France) or Merck (Darmstadt, Germany).

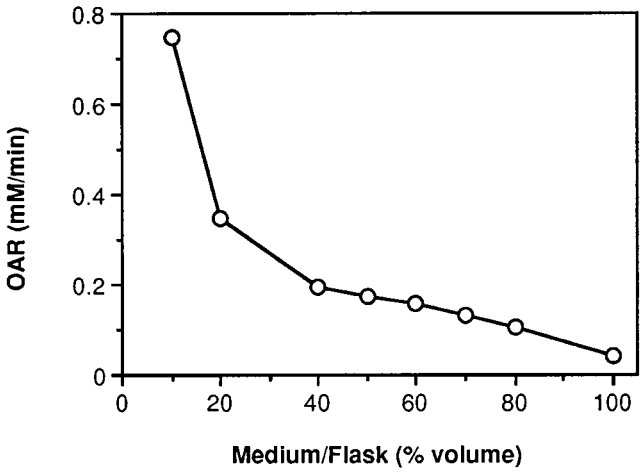


FIG. 1. Aeration efficiency (OAR) in shake flasks containing various volumes of culture medium (volume ratios in percentage of flask volume).

Analysis of the results. The production rate of CP during the exponential growth phase (μ_{CP}, per hour) was calculated from the CP concentration ([CP], in micrograms per milliliter) as follows: $\mu_{CP} = \Delta \ln[CP]/\Delta t$, where t is time.

RESULTS

Type 5 CP production under limited-oxygen conditions. At the high cell densities obtained in fermentor cultures, foaming was intense and became a major technical problem: it was very difficult to maintain the dissolved oxygen at a constant level throughout growth. Most chemical antifoam agents reduce the oxygen transfer rate (20), and addition of such agents decreases the production of extracellular proteins by *S. aureus* (24). To simulate various oxygen concentrations, we chose the previously validated method of filling one size of flask, shaken at the same speed, with different volumes of culture (17, 53). As expected under these conditions, the aeration efficiency was inversely proportional to the volume of culture medium (Fig. 1).

In cultures grown for 24 h (Fig. 2), the reductive capacity of the bacteria decreased steadily with the increasing OAR up to 0.35 mM/min, i.e., 20% volume ratio. Thus, over this volume ratio, growth of *S. aureus* Reynolds in flask culture was under oxygen-limited conditions. Determination of the fermentation products showed that anaerobic-condition-like conditions were obtained with OARs less than 0.13 mM/min, i.e., with volume ratios over 70%. Type 5 CP production by *S. aureus* Reynolds was optimal for OARs over 0.17 mM/min and decreased with decreasing OARs. At OARs less than 0.13 mM/min type 5 CP production was very low (Fig. 2).

Growth and type 5 CP production during culture under limited-oxygen conditions. Growth and type 5 CP production by *S. aureus* Reynolds were monitored in shake flasks with OARs of 0.35, 0.15, and 0.10 mM/min, i.e., under aerobic, oxygen-limited, and anaerobic-condition-like conditions, respectively (Fig. 3A). In all three cultures, exponential growth continued for 3 h, with growth rates (μ) of 0.62, 0.80, and 0.50 h^{-1}, respectively, followed by a slow growth phase. Type 5 CP was produced during the exponential growth phase in the three cultures, with production rates (μ_{CP}) of 0.55, 0.36, and 0.22 h^{-1}, respectively. Only the aerobic and oxygen-limited cultures produced CP during the slow growth phase. In the three cultures, the pH decreased during both the exponential growth and slow growth phases. The pH increased thereafter during the stationary phase only in the aerobic and oxygen-limited cultures (Fig. 3A).

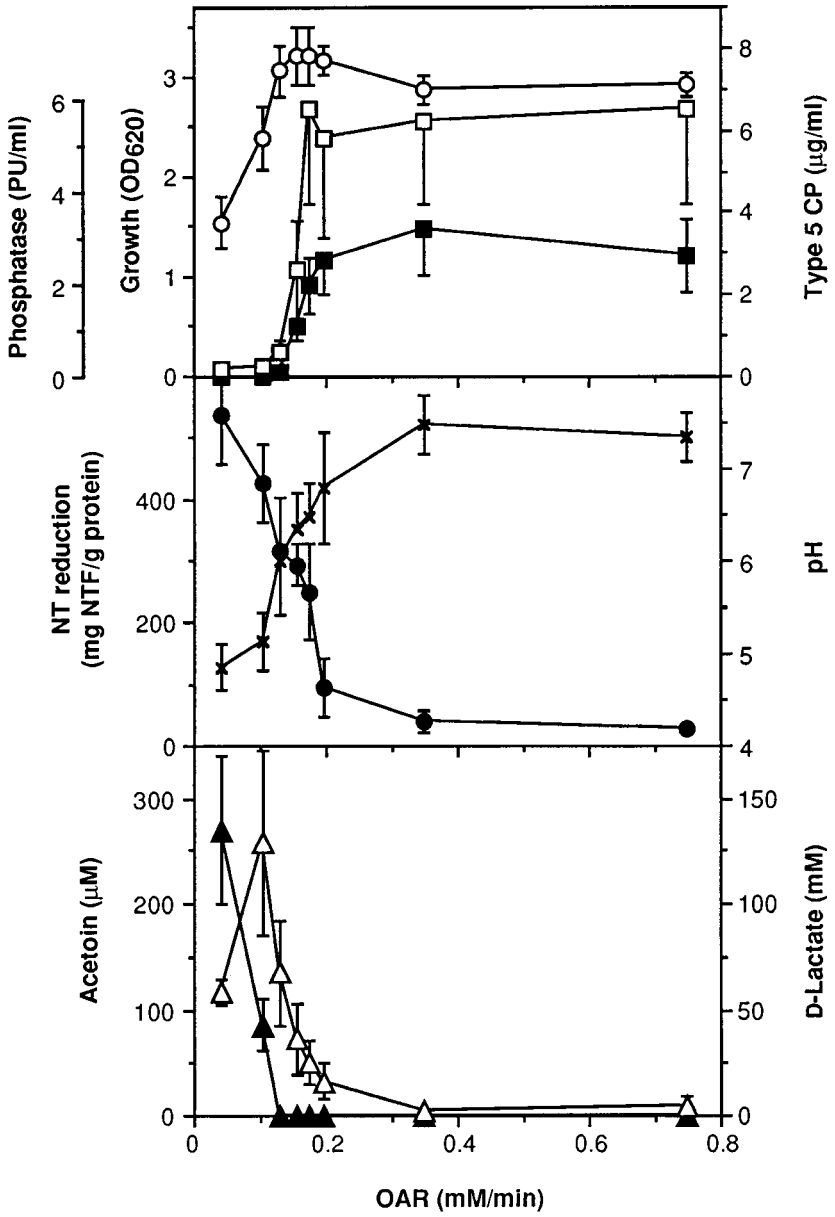
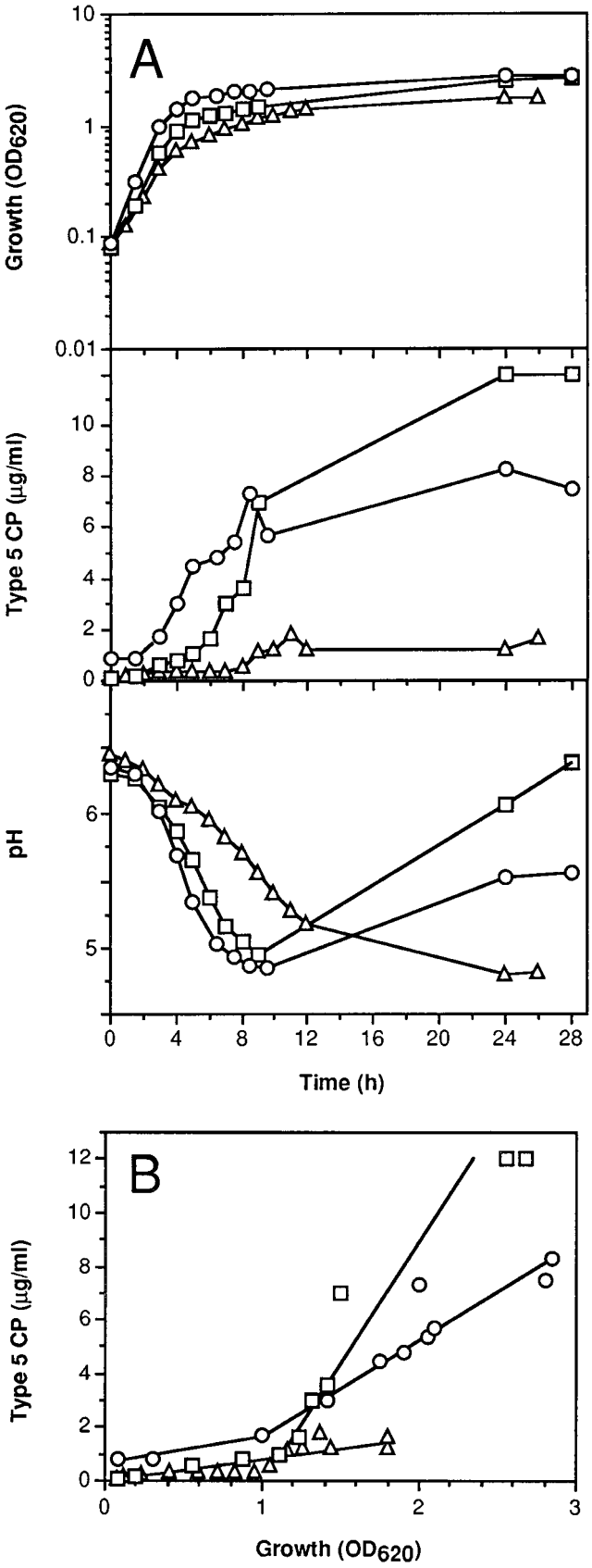


FIG. 2. Effect of aeration efficiency (OAR) on type 5 CP production by *S. aureus* Reynolds grown for 24 h in shake flasks containing various volumes of culture medium. Data are expressed as the mean ± standard error of the mean for four independent experiments. ○, growth; □, type 5 CP; ■, phosphatase; ×, pH; ●, NT reduction; △, acetoin; ▲, D-lactate; PU, units of phosphatase activity.

The slope of CP concentration plotted against growth (Fig. 3B) expresses the differential rate of production (13). This differential rate was the same during the exponential growth phase for the three cultures. It increased at the end of the exponential phase most in the aerobic culture and to a lesser extent in the oxygen-limited culture.

Effect of nitrate respiration on type 5 CP production. In the absence of oxygen, *S. aureus* can use nitrate as a terminal electron acceptor for anaerobic respiration, producing nitrite. In the presence of 8 mM nitrate, *S. aureus* metabolizes more glucose than in aerobic culture and produces a smaller proportion of lactate than in anaerobic culture but more than in aerobic culture (32). In 20 mM nitrate, complete conversion to nitrite is observed (7). These observations indicate that the nitrate concentrations below 20 mM are limiting for *S. aureus* in batch culture. Therefore, we determined type 5 CP production by *S. aureus* Reynolds grown in a fermentor under anaerobic conditions in the presence of 0, 20, and 50 mM nitrate (Table 1). The pH was maintained at 6.5 because post-exponential-phase type 5 CP production was previously observed in the pH range 6 to 7 but not at pH 8 (14). Anaerobic culture without nitrate behaved similarly to the anaerobic-condition-like (90% volume ratio) flask culture, with high lactate production. At 20 mM nitrate, nitrate availability was limiting for *S. aureus*, lactate production was moderate, and there was no post-exponential-phase type 5 CP production. At 50 mM nitrate, nitrate was in excess, there was no lactate production, and type 5 CP was produced in the post-exponential phase (Table 1).

Effects of various metabolic inhibitors on type 5 CP production. *S. aureus* Reynolds was grown in shake flasks under aer-



obic conditions in the presence of subinhibitory concentrations of various metabolic inhibitors (Table 2). These subinhibitory concentrations were fivefold lower than the minimum concentrations that totally inhibited growth of strain Reynolds. TMAO used at concentrations up to 100 mM did not significantly inhibit respiration, growth, or CP production (data not shown). Among the inhibitors tested, only HQNO reproduced the metabolic effects of oxygen deprivation and significantly increased lactate production.

All the inhibitors tested, except TMAO, substantially reduced phosphatase production. There was no post-exponential-phase type 5 CP production in the presence of any of the inhibitors except azide (Table 2). Unlike cyanide and HQNO, azide did not increase the bacterial reductive capacity. Type 5 CP was produced during exponential phase in the presence of all the inhibitors, and μ_{CP} was in all cases lower than μ, although the differential rate of production varied from one inhibitor to another.

DISCUSSION

Capsule production by many bacteria (47, 57), including *S. aureus* (13, 14, 29, 39, 46), is influenced by environmental growth conditions. Our findings confirm the dependence of type 5 CP production by *S. aureus* on the bacterial cell's respiratory activity. Oxygen is subject to concentration fluctuations in the human host (36), and the aerobic respiration of *S. aureus* from tissue fluid is higher than that of bacteria grown in vitro (19). The influence of bacterial respiration on CP production might thus be of some importance in the process of infection. The high level of antibodies against type 5 CP in serum from patients with endocarditis compared with that in patients with septicemia (9) might be explained by the good oxygenation of the cardiac endothelial tissue favoring CP production by infecting *S. aureus*. Moreover, nitric oxide formation is induced in peritoneal macrophages by lipoteichoic acid from *S. aureus*, toxic shock syndrome toxin 1, and killed whole *S. aureus* cells (12) and in endothelial cells by staphylococcal alpha-toxin and enterotoxin B (28, 49). In abscesses, in which completely anaerobic conditions have often been found (36), the presence of host cells stimulated by various microbial products might increase the in situ nitrate concentration (23) and thus CP production by *S. aureus.*

Observations similar to our results have been obtained with coagulase-negative staphylococci. *Staphylococcus epidermidis* produces slime mostly during the post-exponential-growth phase (4, 15), and some strains produce significantly less slime in anaerobic than in aerobic conditions (2, 37). Contradicting our results, Campbell et al. (8) reported that under hypobaric oxygen pressure *S. aureus* can produce slime as a result of impairment of the capacity to oxidize cellular NADH. However, this slime, presented as extrapolymeric substances, has not been chemically characterized, and no relationship between slime production and growth was established. Moreover, considering the ELISA with monoclonal antibodies as a reference method to assess *S. aureus* encapsulation, Sutra et al. (48) reported that diffuse growth in serum-soft agar medium 110 detected 85.5% of encapsulated strains. The lower exposure of the medium to oxygen in the serum-soft agar culture tube

FIG. 3. Growth and type 5 CP production (A) and relationship between type 5 CP production and growth (B) of *S. aureus* Reynolds in shake flasks at different aeration efficiencies (OAR). Data shown are one representative set of data from two separate experiments. □, 0.35 mM/min (20% volume ratio); ○, 0.15 mM/min (60% volume ratio); △, 0.10 mM/min (90% volume ratio).

TABLE 1. Type 5 CP production by *S. aureus* Reynolds grown for 24 h in a fermentor under anaerobic conditions with the pH maintained at 6.5, in the presence of various nitrate concentrations[a]

Nitrate added (mM)	Total growth (OD_{620})	μ (h^{-1})	μ_{CP}[b] (h^{-1})	Nitrite (mM)	NT reduction (mg of NTF/g of protein)	Phosphatase (U/ml)	Type 5 CP production	
							Total (μg/ml)	Post-exponential phase[c]
0	0.83	0.30	0.27	0	693	0.14	0.78	None
20	1.36	0.71	0.65	21	347	0.37	0.90	None
50	1.80	0.72	0.58	36	20	2.14	1.66	Yes

[a] Data shown are one representative set of data from two separate experiments.
[b] Production rate of type 5 CP during the exponential phase of growth, as described in Materials and Methods.
[c] Type 5 CP was always produced during the exponential phase of growth.

could be responsible for the underestimation of encapsulation by this indirect method.

TMAO was not active as a respiratory inhibitor, and this was unexpected because, like HQNO, it possesses a coordinate oxide bond and has been described as an inhibitor of *S. aureus* growth by interacting with the cytochrome *b* of the electron transport system (7, 50). In comparison with cyanide and HQNO, azide is a weak respiratory inhibitor (43) but is a potent inhibitor of the *S. aureus* membrane-associated ATPase activity (27). This might explain the failure of azide to increase the bacterial reductive capacity and the fact that azide reduced total CP production but did not suppress the post-exponential-phase production.

Our results obtained with the respiratory inhibitors demonstrated that type 5 CP production by *S. aureus* is not controlled by an oxygen-dependent regulon like those described for the complex regulation of aerobic and anaerobic cell metabolism (30). However, such oxygen-dependent regulation has been recently implicated in the production of alginate by *Pseudomonas aeruginosa* (31). Our results also contrast with the hypothesis that the large increase in exopolysaccharide synthesis on cessation of growth is due to the release of undecaprenol phosphate, used preferentially for peptidoglycan and teichoic acid synthesis during growth (47, 57). Finally, anaerobic culture with the pH controlled and the effects of azide demonstrate that the post-exponential-phase type 5 CP production is not inhibited by the toxicity of fermentation acids at low pH (41).

Previous attempts to block synthesis of *S. aureus* capsule during the exponential growth phase with 2,4-dinitrophenol were unsuccessful (40). Thus, our results obtained with this ionophore might be interpreted as indicating that post-exponential-phase CP production occurred only when a high enough proton motive force was established by respiratory activity. Under aerobic conditions when the pH is higher than 7.5, the proton motive force decreases to the level observed under anaerobic conditions (26). This is consistent with our previous results showing that post-exponential-phase type 5 CP production was observed in the pH range 6 to 7 but not at pH 8 (14).

The main observation of this study is that whereas type 5 CP was always produced by *S. aureus* during the exponential phase of growth whatever the culture conditions, post-exponential-phase CP production appeared to be strictly dependent on respiratory activity. This suggests that synthesis and polymerization of the sugars making the CP are not dependent on respiratory activity. The energetic requirement for polysaccharide production has been estimated to be less than 1% of the overall ATP turnover during both growth phases (51). Indeed, the cytoplasmic ATP pool is always high enough to allow a basal level of synthesis during growth. The differential rate of this basal exponential-phase type 5 CP production was constant. This is consistent with the observation that type 2 CP is released continuously into the culture fluid at the same rate as cell wall turnover during exponential growth of *S. aureus* Smith (21).

The differential rate of CP production by *S. aureus* increased during the post-exponential phase of growth in batch culture, suggesting that it is under the control of a regulatory system triggered by environmental effectors. It may be that respiratory activity, or possibly a high enough proton motive force, is essential for the induction of post-exponential-phase CP production. We have previously demonstrated (13) that type 5 CP production by *S. aureus* is positively controlled by the accessory gene regulator (*agr*), which induces the secretion of many staphylococcal exoproteins at the end of exponential growth (34). But post-exponential-phase type 5 CP production is not triggered by *agr* and must therefore involve another regulatory system which has not been identified (13). Further studies are

TABLE 2. Type 5 CP production by *S. aureus* Reynolds grown for 24 h in shake flasks, in the presence of various inhibitors at subinhibitory concentrations[a]

Inhibitor added (concn)	Total growth (OD_{620})	μ (h^{-1})	μ_{CP}[b] (h^{-1})	Final pH	NT reduction (mg of NTF/g of protein)	Phosphatase (U/ml)	Type 5 CP production	
							Total (μg/ml)	Post-exponential phase[c]
None	2.79 ± 0.19	0.69 ± 0.12	0.53 ± 0.15	7.37 ± 0.29	27 ± 14	3.46 ± 1.03	6.10 ± 3.40	Yes
Arsenite (0.5 mM)	1.37 ± 0.31	0.63 ± 0.11	0.43 ± 0.12	5.73 ± 0.57	133 ± 60	0	0.18 ± 0.05	None
Cyanide (1 mM)	2.11 ± 0.30	0.31 ± 0.05	0.28 ± 0.09	5.41 ± 0.27	347 ± 75	0	0.56 ± 0.21	None
Azide (2 mM)	1.70 ± 0.14	0.60 ± 0.10	0.27 ± 0.08	5.11 ± 0.06	20 ± 7	0.47 ± 0.10	1.50 ± 0.17	Yes
HQNO (20 μM)	1.38 ± 0.17	0.38 ± 0.07	0.15 ± 0.04	4.66 ± 0.06	907 ± 304	0.15 ± 0.08	0.16 ± 0.09	None
2,4-Dinitrophenol (0.2 mM)	1.71 ± 0.20	0.16 ± 0.03	0.10 ± 0.03	5.81 ± 0.56	27 ± 10	0	1.00 ± 0.60	None

[a] Data are expressed as the mean ± standard error of the mean for four independent experiments.
[b] Production rate of type 5 CP during the exponential growth phase, as described in Materials and Methods.
[c] Type 5 CP was always produced during the exponential phase of growth.

required to characterize the regulation of post-exponential-phase CP production in *S. aureus.*

ACKNOWLEDGMENTS

We thank F. Nato and J.-C. Mazié (Hybridolab, Institut Pasteur) for providing monoclonal antibodies to *S. aureus* type 5 CP.

REFERENCES

1. **Albus, A., J. M. Fournier, C. Wolz, A. Boutonnier, M. Ranke, N. Høiby, H. Hochkeppel, and G. Döring.** 1988. *Staphylococcus aureus* capsular types and antibody response to lung infection in patients with cystic fibrosis. J. Clin. Microbiol. **26:**2505–2509.
2. **Barker, L. P., W. A. Simpson, and G. D. Christensen.** 1990. Differential production of slime under aerobic and anaerobic conditions. J. Clin. Microbiol. **28:**2578–2579.
3. **Barnes, E. H., and J. F. Morris.** 1957. A quantitative study of the phosphatase activity of *Micrococcus pyogenes*. J. Bacteriol. **73:**100–104.
4. **Bayston, R., and J. Rodgers.** 1990. Production of extra-cellular slime by *Staphylococcus epidermidis* during stationary phase of growth: its association with adherence to implantable devices. J. Clin. Pathol. **43:**866–870.
5. **Bochner, B. R., and M. A. Savageau.** 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Appl. Environ. Microbiol. **33:**434–444.
6. **Boutonnier, A., F. Nato, A. Bouvet, L. Lebrun, A. Audurier, J. C. Mazié, and J. M. Fournier.** 1989. Direct testing of blood cultures for detection of the serotype 5 and 8 capsular polysaccharides of *Staphylococcus aureus*. J. Clin. Microbiol. **27:**989–993.
7. **Burke, K. A., and J. Lascelles.** 1975. Nitrate reductase system in *Staphylococcus aureus* wild type and mutants. J. Bacteriol. **123:**308–316.
8. **Campbell, I. M., D. N. Crozier, and A. B. Pawagi.** 1986. Effect of hypobaric oxygen and oleic acid on respiration of *Staphylococcus aureus*. Eur. J. Clin. Microbiol. **5:**622–628.
9. **Christensson, B., A. Boutonnier, U. Ryding, and J. M. Fournier.** 1991. Diagnosing *Staphylococcus aureus* endocarditis by detecting antibodies against *S. aureus* capsular polysaccharide type 5 and 8. J. Infect. Dis. **163:** 530–533.
10. **Coleman, G., I. T. Garbutt, and U. Demnitz.** 1983. Ability of a *Staphylococcus aureus* isolate from a chronic osteomyelitic lesion to survive in the absence of air. Eur. J. Clin. Microbiol. **2:**595–597.
11. **Cross, A. S.** 1990. The biological significance of bacterial encapsulation. Curr. Top. Microbiol. Immunol. **150:**87–95.
12. **Cunha, F. Q., D. W. Moss, L. M. C. C. Leal, S. Moncada, and F. Y. Liew.** 1993. Induction of macrophage parasiticidal activity by *Staphylococcus aureus* and exotoxins through the nitric oxide synthesis pathway. Immunology **78:**563–567.
13. **Dassy, B., T. Hogan, T. J. Foster, and J. M. Fournier.** 1993. Involvement of the accessory gene regulator (*agr*) in expression of type 5 capsular polysaccharide by *Staphylococcus aureus*. J. Gen. Microbiol. **139:**1301–1306.
14. **Dassy, B., W. T. Stringfellow, M. Lieb, and J. M. Fournier.** 1991. Production of type 5 capsular polysaccharide by *Staphylococcus aureus* grown in a semisynthetic medium. J. Gen. Microbiol. **137:**1155–1162.
15. **Deighton, M., and R. Borland.** 1993. Regulation of slime production in *Staphylococcus epidermidis* by iron limitation. Infect. Immun. **61:**4473–4479.
16. **Fournier, J. M.** 1991. *Staphylococcus aureus*, p. 166–177. *In* S. J. Cryz, Jr. (ed.), Vaccines and immunotherapy. Pergamon Press, New York.
17. **Freedman, D.** 1970. The shaker in bioengineering. Methods Microbiol. **2:**175–185.
18. **Gardner, J. F., and J. Lascelles.** 1962. The requirement for acetate of a streptomycin-resistant strain of *Staphylococcus aureus*. J. Gen. Microbiol. **29:**157–164.
19. **Gellenbeck, S. M.** 1962. Aerobic respiratory metabolism of *Staphylococcus aureus* from an infected animal. J. Bacteriol. **83:**450–455.
20. **Ghildyal, N. P., B. K. Lonsane, and N. G. Karanth.** 1988. Foam control in submerged fermentation: state of the art. Adv. Appl. Microbiol. **33:** 173–222.
21. **Havaei, S. A., and I. C. Hancock.** 1994. The capsular turnover product of *Staphylococcus aureus* strain Smith. FEMS Microbiol. Lett. **118:**37–43.
22. **Hochkeppel, H. K., D. G. Braun, W. Vischer, A. Imm, J. Sutter, U. Staeubli, R. Guggenheim, E. L. Kaplan, A. Boutonnier, and J. M. Fournier.** 1987. Serotyping and electron microscopy studies of *Staphylococcus aureus* clinical isolates with monoclonal antibodies to capsular polysaccharide types 5 and 8. J. Clin. Microbiol. **25:**526–530.
23. **Jacob, T. D., D. K. Nakayama, I. Seki, R. Exler, J. R. Lancaster, M. A. Sweetland, S. Yousem, R. L. Simmons, T. R. Billiar, A. B. Peitzman, and E. K. Motoyama.** 1994. Hemodynamic effects and metabolic fate of inhaled nitric oxide in hypoxic piglets. J. Appl. Physiol. **76:**1794–1801.
24. **Jarvis, A. W., R. C. Lawrence, and G. G. Pritchard.** 1973. Production of staphylococcal enterotoxins A, B, and C under conditions of controlled pH and aeration. Infect. Immun. **7:**847–854.
25. **Karakawa, W. W., J. M. Fournier, W. F. Vann, R. Arbeit, R. S. Schneerson, and J. B. Robbins.** 1985. Method for the serological typing of the capsular polysaccharides of *Staphylococcus aureus*. J. Clin. Microbiol. **22:**445–447.
26. **Kashket, E. R.** 1981. Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. J. Bacteriol. **146:**369–376.
27. **Kubak, B. M., and W. W. Yotis.** 1981. *Staphylococcus aureus* adenosine triphosphatase: inhibitor sensitivity and release from membrane. J. Bacteriol. **146:**385–390.
28. **LeClaire, R. D., W. M. Kell, R. A. Sadik, M. B. Downs, and G. W. Parker.** 1995. Regulation of staphylococcal enterotoxin B-elicited nitric oxide production by endothelial cells. Infect. Immun. **63:**539–546.
29. **Lee, J. C., S. Takeda, P. J. Livolsi, and L. C. Paoletti.** 1993. Effects of in vitro and in vivo growth conditions on expression of type 8 capsular polysaccharide by *Staphylococcus aureus*. Infect. Immun. **61:**1853–1858.
30. **Lin, E. C. C., and S. Iuchi.** 1991. Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. Annu. Rev. Genet. **25:**361–387.
31. **Mohr, C. D., S. K. Sonsteby, and V. Deretic.** 1994. The *Pseudomonas aeruginosa* homologs of *hemC* and *hemD* are linked to the gene encoding the regulator of mucoidy AlgrR. Mol. Gen. Genet. **242:**177–184.
32. **Morse, S. A., and R. A. Mah.** 1973. Regulation of staphylococcal enterotoxin B: effect of anaerobic shock. Appl. Microbiol. **25:**553–557.
33. **Nicholas, D. J. D., and A. Nason.** 1957. Determination of nitrate and nitrite. Methods Enzymol. **3:**981–984.
34. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh.** 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. **248:**446–458.
35. **Nychas, G. J., H. S. Tranter, R. D. Brehm, and R. G. Board.** 1991. *Staphylococcus aureus* S-6: factors affecting its growth, enterotoxin B production and exoprotein formation. J. Appl. Bacteriol. **70:**344–350.
36. **Park, M. K., R. A. M. Myers, and L. Marzella.** 1992. Oxygen tensions and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin. Infect. Dis. **14:**720–740.
37. **Pérez-Giraldo, C., A. Rodríguez-Benito, F. J. Morán, C. Hurtado, M. T. Blanco, and A. C. Gómez-García.** 1995. Influence of the incubation atmosphere on the production of slime by *Staphylococcus epidermidis*. Eur. J. Clin. Microbiol. Infect. Dis. **14:**359–362.
38. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83:**346–356.
39. **Poutrel, B., F. B. Gilbert, and M. Lebrun.** 1995. Effects of culture conditions on production of type 5 capsular polysaccharide by human and bovine *Staphylococcus aureus* strains. Clin. Diagn. Lab. Immunol. **2:**166–171.
40. **Powelson, D. M., P. W. Wilson, and R. H. Burris.** 1947. Oxidation of glucose, glycerol and acetate by *Staphylococcus aureus*. Biochem. J. **41:**486–491.
41. **Russell, J. B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. **73:**363–370.
42. **Sheagren, J. N.** 1984. *Staphylococcus aureus*: the persistent pathogen. N. Engl. J. Med. **310:**1368–1373, 1437–1442.
43. **Smith, L.** 1954. Bacterial cytochromes. Bacteriol. Rev. **18:**106–130.
44. **Smith, S. M., R. H. Eng, and F. Buccini.** 1986. Use of D-lactic acid measurements in the diagnosis of bacterial infections. J. Infect. Dis. **154:**658–664.
45. **Strasters, K. C., and K. C. Winkler.** 1963. Carbohydrate metabolism of *Staphylococcus aureus*. J. Gen. Microbiol. **33:**213–229.
46. **Stringfellow, W. T., B. Dassy, M. Lieb, and J. M. Fournier.** 1991. *Staphylococcus aureus* growth and type 5 capsular polysaccharide production in synthetic media. Appl. Environ. Microbiol. **57:**618–621.
47. **Sutherland, I. W.** 1988. Bacterial surface polysaccharides: structure and function. Int. Rev. Cytol. **113:**187–231.
48. **Sutra, L., C. Mendola, P. Rainard, and B. Poutrel.** 1990. Encapsulation of *Staphylococcus aureus* isolates from mastitic milk: relationship between capsular polysaccharide types 5 and 8 and colony morphology in serum-soft agar, clumping factor, teichoic acid, and protein A. J. Clin. Microbiol. **28:** 447–451.
49. **Suttorp, N., M. Fuhrmann, S. Tannert-Otto, F. Grimminger, and S. Bhakdi.** 1993. Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. J. Exp. Med. **178:**337–341.
50. **Suzuki, S., A. Kubo, H. Shinano, and K. Takama.** 1992. Inhibition of the electron transport system in *Staphylococcus aureus* by trimethylamine-N-oxide. Microbios **71:**145–148.
51. **Tempest, D. W., and O. M. Neijssel.** 1984. The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. Annu. Rev. Microbiol. **38:**459–486.
52. **Thom, S. M., R. W. Horobin, E. Seidler, and M. R. Barer.** 1993. Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. J. Appl. Bacteriol. **74:**433–443.
53. **Turner, W. H.** 1978. The effect of medium volume and yeast extract diffusate on δ-hemolysin production by five strains of *Staphylococcus aureus*. J. Appl. Bacteriol. **45:**291–296.
54. **Urban, T., and C. Jarstrand.** 1979. Nitroblue tetrazolium (NBT) reduction

by bacteria: some properties of the reaction and its possible use. Acta Pathol. Microbiol. Scand. Sect. B **87:**227–233.
55. **Vistica, D. T., P. Skehan, D. Scudiero, A. Monks, A. Pittman, and M. R. Boyd.** 1991. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Res. **51:**2515–2520.
56. **Westerfeld, W. W.** 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161:**495–502.
57. **Whitfield, C., and M. A. Valvano.** 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. Adv. Microb. Physiol. **35:**135–246.
58. **Wilkinson, B. J.** 1983. Staphylococcal capsules and slime, p. 481–523. *In* C. S. F. Easmon and C. Adlam (ed.), Staphylococci and staphylococcal infections, vol. 2. Academic Press, Inc., New York.

Editor: V. A. Fischetti