On the production of staphylocoagulase by staphylococcus aureus

Citation for published version (APA):

Engels, W. (1981). *On the production of staphylocoagulase by staphylococcus aureus*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg. <https://doi.org/10.26481/dis.19811218we>

Document status and date:

Published: 01/01/1981

DOI:

[10.26481/dis.19811218we](https://doi.org/10.26481/dis.19811218we)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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on the production of staphylocoagulase by staphylococcus aureus

P R O E F S C H R I F T

ter verkrijging van de graad van
Doctor in de Geneeskunde aan de
Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus
Prof.Dr. W.H.F.W. Wijnen,
volgens het besluit van het College van Dekanen
in het openbaar te verdedigen
in de Aula van de Universiteit
op vrijdag, 18 december 1981
des namiddags te 16.00 uur

door

willem engels

geboren te Helmond in 1946

PROMOTOR : Prof.Dr. C.P.A. van Boven

REFERENTEN: Prof.Dr. H.C. Hemker
Prof.Dr. D.W. Tempest
Prof.Dr. J. Verhoef

Dit proefschrift werd bewerkt in het laboratorium voor
Medische Microbiologie van de Rijksuniversiteit Limburg,
onder leiding van Prof.Dr. C.P.A. van Boven.

The study of the growth of bacterial cultures does not constitute a specialized subject or a branch of research: it is the basic method of Microbiology.

Jacques Monod (1949)

Aan mijn ouders

Manuscript : Mevr. F. Claus-Hahn
Tekeningen : Mevr. M.A.F. Kamps
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Rijksuniversiteit Limburg

CONTENTS

Voorwoord

Abbreviations

Chapter 1 Introduction

Chapter 2 Influence of cultivation conditions on the production of staphylocoagulase by Staphylococcus aureus 104. (J. Gen. Microbiol. (1978) 109: 237-243)

Chapter 3 Continuous culture studies on the production of staphylocoagulase by Staphylococcus aureus. (Antonie van Leeuwenhoek (1980) 46: 533-550)

Chapter 4 Secretion of staphylocoagulase by Staphylococcus aureus: the role of a cell-bound intermediate. (Antonie van Leeuwenhoek, accepted for publication)

Chapter 5 The competitive ability of spontaneous staphylocoagulase-negative mutants of Staphylococcus aureus 104 and NCTC 8178 with respect to growth of the parent strains in continuous culture. (Antonie van Leeuwenhoek, accepted for publication; in abstract form, Antonie van Leeuwenhoek 47: 90-91)

Chapter 6 Rapid and direct staphylocoagulase assay that uses a chromogenic substrate for identification of Staphylococcus aureus. (J. Clin. Microbiol. (1981) 13: 496-500)

Chapter 7 General discussion and Summary

Samenvatting

VOORWOORD

Het verschijnen van dit proefschrift stelt mij in de gelegenheid allen te bedanken die hieraan een directe of indirecte bijdrage hebben geleverd. Zonder daarbij iemand tekort te willen doen, wil ik een aantal met name noemen.

Allereerst ben ik grote dank verschuldigd aan mijn ouders, die mij in staat hebben gesteld een universitaire opleiding te volgen. Hun voortdurende interesse is een belangrijke stimulans geweest voor mijn verdere ontwikkeling.

Prof.Dr. C.P.A. van Boven. Cees, ik ben je zeer erkentelijk voor de gelegenheid die je mij hebt geboden voor het uitvoeren van dit onderzoek. Ondanks je drukke facultaire werkzaamheden vond je steeds tijd om het onderzoek met grote interesse en kritische opmerkingen te begeleiden.

Prof.Dr. H.C. Hemker dank ik voor zijn initiatief tot het stafylocoagulase-onderzoek en de manier waarop hij mij heeft weten te enthousiasmeren voor dit curieuze eiwit.

Prof.Dr. D.W. Tempest. Dave, jouw ideeën over fenotypische variabiliteit van micro-organismen hebben mede de basis gevormd voor dit proefschrift. Voor de wijze waarop je dit proefschrift aan je kritiek hebt onderworpen, ben ik je zeer erkentelijk.

Prof.Dr. J. Verhoef dank ik voor zijn belangstelling voor dit onderzoek en de geboden hulp bij het uitvoeren van fagocytose experimenten.

Dr. O.M. Neijssel. Oense, het voorliggend proefschrift is een voorbeeld van een goede interuniversitaire samenwerking. Niet alleen onze geregelde discussies, doch ook de vele, vaak telefonische, adviezen hebben steeds een stimulerende invloed gehad. Jouw optimisme en kritische aanpak hebben geleid tot het verkregen resultaat. Ook wil ik je bedanken voor de enorme steun bij het corrigeren van de vele manuscripten.

Dr. J.T.M. Wouters. Jan, jouw kennis op het gebied van celwanden en competitie experimenten in continue cultuur waren van onschatbare waarde voor dit onderzoek. Ik hoop dat je jouw aandeel herkent in dit proefschrift.

Drs. W.J. van Leeuwen ben ik zeer dankbaar voor het karakteriseren van de vele S. aureus stammen en de isolatie van diverse plasmiden.

Miriam Kamps ben ik zeer dankbaar voor de uitvoering van de vele experimenten en het tekenen van de figuren.

Ik besef dat mijn, soms chaotische, manier van werken je dikwijls tot wanhoop heeft gebracht. Zonder je kritische en nauwgezette werkwijze zou dit onderzoek niet mogelijk zijn geweest.

Puck Muller dank ik voor het oplossen van vele stolperikelen en het standaardiseren van humaan PPSB.

Hans Hendrix dank ik voor zijn hulp bij de zuivering van stafylocoagulase.

Fia Claus dank ik voor de enthousiaste wijze waarop zij met veel geduld mijn hiërogliefen steeds weer in voortreffelijk type-werk heeft weten om te zetten.

Jacques Beckers, Michel Beesmans en Gerry Tilly worden bedankt voor de sterilisatie van de kweekapparatuur en de 4182 liter kweekmedium.

De medewerkers van de Algemene Instrumentele Dienst dank ik voor het modificeren van de kweekapparatuur.

Tenslotte gaat mijn waardering uit naar alle collega's van het Biomedisch Centrum, omdat zij altijd bereid waren medewerking in welke vorm dan ook te verlenen.

ABBREVIATIONS

BHI	Brain Heart Infusion
BSA	Bovine serum albumin
D	Dilution rate
DNA	Deoxyribonucleic acid
DOC	Dissolved oxygen concentration
EDTA	Ethylene diaminetetraacetic acid
EMS	Ethyl methanesulphonate
K_s	Growth-limiting substrate concentration giving half-maximal growth rate
LD ₅₀	Medium dose that kills 50% of the animals
μ	Specific growth rate
NB	Nutrient broth
PBS	Phosphate buffered saline
PPSB	Prothrombin, proconvertin, Stuart-Prower factor, antihaemophilic factor B
QO ₂	μ l oxygen consumed per mg dry wt organisms and per hour
qO ₂	μ mol oxygen consumed per mg dry wt organisms and per hour
RNA	Ribonucleic acid
SC	Staphylocoagulase
TEP	Total extracellular protein
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

Staphylococcus aureus is a remarkably versatile species, which has been very successful in establishing itself as a commensal in various niches on the human and animal body (55).

Staphylococcal carriage is present in 20-40% of the general human population whereas hospital personnel and patients often have higher carrier rates, up to 90% (31, 35, 83). In the latter groups, these staphylococci are often resistant to one or more antibiotics in use. S. aureus is located primarily in the anterior nares and to a lesser extent on the perineum. In 70-90% of the population S. aureus can be isolated transiently from at least one site (generally the skin) (26, 84). Although in the large majority of cases this carriage state is one of peaceful commensalism (83), it is clear that carriers of staphylococci are at greater risk of acquiring infective lesions (19), since there is no evidence that the carriage of staphylococci in the nose or on the skin leads to immunity to staphylococcal infections (23). Besides patients with staphylococcal lesions, asymptomatic carriers are potential sources of hospital-acquired S. aureus infections. Generally, there is a steady increase in the acquisition of staphylococci, the longer a patient stays in hospital.

Many reports agree that S. aureus is not usually dispersed by droplet transmission but rather is disseminated by transfer of the organisms from the nose or perineum to the skin, clothing and bedding of the carrier. From these areas there is a potential of S. aureus being spread, particularly by hand contact to other persons or inanimate sources (58). Furthermore the development of nasal carriage seems to correlate better with the epidemic virulence of a staphylococcal strain than with air contamination. In general, disinfection of the skin reduces cross-infections more effectively than nasal disinfection (66). Cross-infection from one human to another is extremely common; it is sometimes airborne but in the majority of cases the result of direct or indirect contact. The ubiquity of staphylococci in the population is such that elimination of the human reser-

voir would appear virtually impossible.

Although in the general population serious infections due to S. aureus are relatively rare, they continue to be a major source of morbidity and mortality in a hospital-based practice. S. aureus can interfere with surgical wound healing, cause septicemia and endocarditis, colonize burns and is often the cause of osteomyelitis and furunculosis, especially in the immune-compromised host. In the last two decades the number of these susceptible patients has increased considerably (9, 57).

Intact skin and slime layers are generally thought to be the primary defence against soft tissue infections by S. aureus (which are the most in common) (26), although in humans it has been surprisingly difficult, even after breaking the skin, to produce experimental infections (16, 51, 67).

Once the outer barriers have been breached, phagocytosis and intracellular killing of the staphylococcus by leucocytes represent the major line of defence of the host against infection. These two processes require complex interactions between bacterial cell surfaces and soluble factors in serum (opsonins) on the one hand and phagocytic cells on the other (78). Recurrent or severe infections may result from a defective host response in any step of these complex interactions (57, 62, 79).

The virulence of S. aureus is based on cell-surface components such as peptidoglycan, protein A, teichoic acid, capsular material (24, 60, 80) and a large number of extracellular proteins, which are believed to have biologic effects on the intended host (1, 64, 86). Because of a high correlation between the virulence factors of S. aureus and the observed pathogenicity in man, many attempts have been made to relate staphylococcal virulence to these factors. Few of these factors have been demonstrated as a pathogenic link between the staphylococcus and staphylococcal disease (1, 57). With respect to the exoproteins, staphylocoagulase has been found to accompany pathogenicity most consistently, although virulent staphylocoagulase-negative as well as avirulent staphylocoagulase-positive strains have been reported (1, 4, 23, 26).

In addition, the ability to produce staphylocoagulase is by definition one of the main criteria used to distinguish S. aureus from other staphylococci (5). Although staphylocoagulase-negative staphylococci were generally considered as non-pathogenic commensal bacteria of the human skin, there is an increasing awareness during the last ten years that these organisms can cause a variety

of infections including bacteremia, endocarditis, infections associated with foreign bodies such as ventriculo-atrial shunts and cardiac valve prostheses, and especially infections of the urinary tract (59).

This chapter will review the mode of action of staphylocoagulase and its role in the pathogenesis of S. aureus. The objectives of the thesis are further delineated in the last section of this chapter.

1.1 PURIFICATION AND MODE OF ACTION OF STAPHYLOAGULASE

In 1903 Loeb (49) demonstrated the specific action of S. aureus in clotting goose plasma in vitro and he proposed a correlation between the clotting ability of staphylococci and pathogenicity. These findings were confirmed by Munch (56). Later investigations of Gratia (32) revealed that the coagulation was due to a protein excreted by the organism. This protein was called staphylocoagulase.

Studies of Smith and Hale (68) provided evidence that besides fibrinogen, a second plasma factor was required for the action of staphylocoagulase. This substance, first called activator by Smith and Hale, was labeled Coagulase Reacting Factor (CRF) by Tager (73).

It was proposed that CRF was identical to prothrombin and that a complex between staphylocoagulase and prothrombin was formed. Subsequently, this complex would be able by virtue of thrombin-like activity to convert fibrinogen into fibrin, the actual coagulation reaction (74). Neither the coagulation nor the esterase activity of this complex is inhibited by heparin, hirudin or antithrombin III which are potent inhibitors of thrombin activity (70).

Since 1948 a large number of reports have been published dealing with the purification of staphylocoagulase, but with almost all of these preparations none were of sufficient homogeneity to assess the mode of action of staphylocoagulase.

Duthie (18) showed that S. aureus produced two forms of staphylocoagulase: a free staphylocoagulase which was secreted into the cultivation medium and a bound-coagulase. However, the latter differs completely from free staphylocoagulase with respect to mode of action and antigenicity and was therefore called clumping factor (18, 74).

With a highly purified preparation of staphyloco-

agulase Hemker and co-workers (7, 36) characterized this protein and resolved its mode of action. Staphylocoagulase, produced by S. aureus strain 104 is a single chain protein with a molecular weight of 61000, aspartic acid as the N-terminal amino acid and an isoelectric point of 4.53. The protein does not contain (phospho)-lipid or carbohydrate residues.

The activation of human prothrombin by staphylocoagulase occurs stoichiometrically, one molecule of staphylocoagulase forming a complex with one of prothrombin without splitting of peptide bonds. This complex i.e. staphylothrombin has antigenic determinants in common with prothrombin and staphylocoagulase but not with thrombin. Moreover, the amino acid composition of staphylothrombin equals the sum of the amino acid compositions of staphylocoagulase and prothrombin. Therefore, the thrombin-like activity of this complex is probably not due to proteolytic breakdown of prothrombin to thrombin but to a change of the tertiary structure of prothrombin that unmasks a thrombin-like active centre. The coagulation reaction brought about by staphylocoagulase is independent of Ca^{2+} ions, phospholipids and other blood clotting factors whereas staphylocoagulase by itself does not possess proteolytic or esterase activity.

Similar results were obtained by Zajdel et al. (87) who have used a staphylocoagulase preparation produced by a bovine strain of S. aureus. The changes brought about by thrombin and staphylothrombin in the substrate fibrinogen are identical as indicated by the change in nitrogen percentage, structure of the individual fibrinogen peptides and the endgroups of the fibrin monomers (87).

Recently Lindhout et al. (47) demonstrated with purified staphylocoagulase, prepared by a bovine prothrombin-sepharose chromatography column, that staphylocoagulase and prothrombin form a firm, equimolar complex, i.e. staphylothrombin with a dissociation constant less than 10^{-11} M. Furthermore, they confirmed the observation of Hemker et al. (36) that proteolysis cannot account for the generation of thrombin-like activity. The thrombin-like activity can be generated as a result of the interactions between staphylocoagulase and human prethrombin 1, human prethrombin 2 or bovine prethrombin 2. Interaction between staphylocoagulase and thrombin give a complex in which the thrombin activity cannot be inhibited by antithrombin III and hirudin. Finally, staphylothrombin does not possess activity

towards factor V, factor VIII and platelets. Kawabata et al. (43) also demonstrated that staphylocoagulase binds in the prethrombin-2 region of prothrombin.

During administration of vitamin K antagonists, vitamin K-dependent coagulation factors enter the plasma in an abnormal form as the so-called "proteins induced by vitamin K absence" (PIVKA's) (37). It has been postulated that the discrepancy between the assays for CRF and prothrombin during anticoagulant treatment is due to the fact that although PIVKA-II has no activity in the normal tests for prothrombin, it does have a CRF activity (39). Bas et al. (8) demonstrated that staphylocoagulase not only reacts with normal prothrombin but also with the precursor of prothrombin, PIVKA-II. Therefore, staphylocoagulase finds a new application in the determination of PIVKA-II during anticoagulant therapy.

Intravenous application of staphylocoagulase in a sub-lethal dose in rabbits results, after three hours, in a decrease of the fibrinogen level to about 55% of the initial value; the levels of factor V and factor VIII are not affected by staphylocoagulase. After 16 hours, the level of fibrinogen rises by about 200% whereas the levels of the factors V and VIII were increased by 40% (87). A lethal dose of staphylocoagulase results in a rapid defibrination and the death of the rabbit in 10-20 min after the intravenous injection, due to fibrin deposits in kidney capillaries, adrenals, liver and small and large lung vessels (69, 71, 87).

Using literature data, Elek (26) presented conclusive evidence that staphylocoagulase is antigenic and that antibodies to the antigen can be raised in vivo. At least four antigenically distinct staphylocoagulases have been demonstrated (17, 63). A close relationship between the bacteriophage group of staphylococcal strains and the antigenic specificity of the staphylocoagulase produced by the corresponding strains was observed by Barber and Wildy (6). In addition, staphylococci of phage type 3A (group II) and 42E (group III) produce staphylocoagulases which are antigenically distinct from those produced by other strains of group II and III, respectively.

The observations of Barby and Wildy were confirmed by Blobel and Berman (10), but the differences in the antigenicity of the staphylocoagulases did not appear to be sufficiently distinct to justify a classification of staphylococci for epidemiologic investigation.

Nevertheless, Zen-Yoji et al. (88) reported that staphylocoagulase existed in seven antigenically dis-

distinct different types and that staphylocoagulase-positive staphylococci could be classified by staphylocoagulase typing (89). Recently, Ushioda and co-workers (76) were able to classify almost all staphylocoagulase-positive staphylococci into eight types by staphylocoagulase typing. Following this method, Dekio and Onimura (15) indicated that relationships were obviously present between the various dermatoses and the staphylocoagulase types of the S. aureus strains isolated from their lesions.

Up to this point, all evidence indicated that a single S. aureus strain produces a single antigenic type of staphylocoagulase. However, Henderson and Brodie (38) found that a given strain could produce either a single staphylocoagulase or as many as four antigenically distinct types, suggesting heterogeneity or multiple molecular forms of the protein. Miale et al. (54) demonstrated even five so-called staphylococcal isocoagulases. However, when staphylocoagulase was purified more extensively, this number had decreased considerably.

Generally, staphylocoagulase reacts not only with plasma of the animal or human species from which the corresponding S. aureus strain is isolated, but also with plasma of other species. Although this phenomenon has been reported repeatedly in the literature, no explanation was given (33, 48, 85). With the present knowledge of the mode of action of staphylocoagulase, it is very likely that every staphylocoagulase has a certain specificity towards prothrombin of various species.

1.2 ROLE OF STAPHYLOCOAGULASE IN THE PATHOGENESIS OF S. AUREUS

In 1945, Hale and Smith (34) suggested that the production of staphylocoagulase protected S. aureus from phagocytosis. Staphylocoagulase-positive staphylococci were poorly phagocytized when suspended in citrated blood, which contains fibrinogen, whereas rapid phagocytosis occurred in defibrinated blood. On the other hand staphylocoagulase-negative staphylococci were phagocytized with equal avidity by polymorphonuclear leucocytes, irrespective of whether they were suspended in citrated or defibrinated blood. Furthermore, it was demonstrated in stained films, that the plasma-treated staphylocoagulase-positive staphylococci were surrounded by a fibrin deposit. Therefore, it was concluded that staphylocoagulase forms a barrier of fibrin around the bacteria which protects them from phagocytosis.

Cawdery et al. (13) disputed the results of Smith and Hale. They showed that the process of defibrination resulted in a significant loss of leucocytes from the blood and that therefore the number of staphylococci phagocytized was greater in defibrinated than in citrated blood. When the number of leucocytes was adjusted so that the counts in the defibrinated and citrated blood were equal, no difference was found in the numbers of staphylocoagulase-positive and staphylocoagulase-negative staphylococci phagocytized. Although several authors reported that sera of patients, suffering from chronic staphylococcal infections, contain high titres of antibodies to staphylocoagulase, protection experiments by immunization with staphylocoagulase against an experimental staphylococcal infection give non-consistent results (21, 23).

Melly et al. (53) observed that staphylocoagulase-positive staphylococci survived in small numbers within the cytoplasm of human granulocytes for prolonged periods of time whereas staphylocoagulase-negative staphylococci appeared to be destroyed rapidly.

When staphylocoagulase-negative strains of S. aureus were suspended in solutions of partially purified staphylocoagulase and injected intracerebrally into mice, a significant mortality resulted; organisms or staphylocoagulase alone injected at the same dose level were shown to be innocuous (25). However, studies of Kapral and co-workers (41, 42, 46) and of Van der Vijver et al. (77) with staphylocoagulase-negative mutants revealed no difference in virulence between the wild type and the mutant strains.

Another role of staphylocoagulase as a virulence factor of S. aureus was proposed by Bullen et al. (12). Many staphylocoagulase-positive strains of S. aureus grow readily in serum while staphylocoagulase-negative strains grow much more slowly if at all. The addition of purified staphylocoagulase to serum abolished the inhibitory effect (22). Schadé (65) demonstrated that the growth rate of S. aureus in human serum was directly correlated with the percentage of iron-saturation of transferrin and that S. epidermidis did not grow in normal serum unless transferrin was saturated with iron. From these observations, Bullen et al. concluded that staphylocoagulase might possibly be involved in making iron available from transferrin to the bacterial cells.

This idea was supported by experiments of Marcelis et al. (50) in which it was shown that the growth of S. epidermidis could be inhibited completely by the

addition of transferrin whereas the growth of S. aureus was not affected. However, it cannot be concluded that the observed phenomena were due exclusively to staphylocoagulase since both S. aureus and S. epidermidis produce iron-chelating substances (50).

Apart from a possible role of staphylocoagulase in the release of iron from transferrin, it has to be stressed that the availability of iron for micro-organisms during the infection process is of great importance. A large number of publications have revealed the vigorous competition for growth-essential iron between micro-organisms and their hosts. The latter possess an array of mechanisms to withhold iron; the ability of micro-organisms to overcome these mechanisms is an important component of virulence (29, 82).

From these studies it can be concluded that the mechanism by which staphylocoagulase may increase the persistence of S. aureus during the infection process is by no means understood and that a correlation between the ability to produce staphylocoagulase and the virulence of S. aureus has not been established. Therefore it is now assumed that in general the virulence of a S. aureus strain is determined by a combination of factors.

1.3 INTRODUCTION TO THE THESIS

S. aureus is an extremely adaptable micro-organism capable of rapidly changing its genotypic and phenotypic characteristics (55). The development of staphylococcal resistance to many antimicrobial agents which has led to a world-wide increase of multiresistant hospital strains during the period 1940-1960 is an example of this adaptability (45, 61).

Not only the composition of the cell-wall but also the amount and variety of the extracellular proteins are strongly dependent upon growth conditions (3, 4, 11, 30). Therefore by changing its antigenic structure and opsonic requirements, S. aureus can present a different "face" to the host, leading to a less efficient phagocytosis and subsequently to a delayed healing process (24). Thus, the virulence of S. aureus may vary according to the environment in which the staphylococci grow (2, 29, 40). Consequently, the initial stage and the course of the staphylococcal infections will be greatly influenced by environmental conditions. This is illustrated by two examples: staphylococci in vivo are more resistant to intracellular killing by polymorphonuclear

leucocytes than organisms grown in vitro (2); S. aureus strains which failed to produce α -haemolysin and leucocidin in vitro, produced high amounts of these enzymes in vivo (28). However, few data are available about the environmental conditions under which S. aureus grows in vivo.

Gladstone and Glennocross (28) showed that the growth rate of S. aureus in vivo had decreased considerably compared to that in vitro. Moreover, they pointed out that S. aureus grew under oxygen-limited growth conditions in their experiments.

From recent studies, it can be calculated that, in vivo, S. aureus multiplies with growth rates in the range of $0.12-0.35 \text{ h}^{-1}$ (generation times of 2-6 hours) (14, 44, 81). Thus, it is likely that, in vivo, S. aureus will be growing at submaximal rates, presumably as a consequence of substrate-limited growth conditions.

Since staphylocoagulase production is still considered to accompany pathogenicity of S. aureus most consistently, the production of this exoprotein under these growth conditions was chosen for further study. The following aspects of staphylocoagulase production by S. aureus were investigated in this thesis:

1. Studies in batch cultures revealed that the production by S. aureus is dependent on strain type, composition of the cultivation medium, temperature, inoculum size, growth phase, pH and aeration efficiency (20, 27, 52, 72, 75). Therefore a new investigation was undertaken to assess the effects of medium composition and environmental factors on the formation of staphylocoagulase by S. aureus strain 104 when grown in batch cultures. This strain was chosen because high yields of staphylocoagulase had been obtained with it previously (Chapter 2).
2. Since, in contrast to a batch culture, the chemostat provides a defined and reproducible environment in which micro-organisms can grow under steady-state conditions at submaximal rates, continuous cultures with strain 104 and with two bovine strains of S. aureus were set up and the effect of various environmental parameters on their production of staphylocoagulase was studied (Chapter 3).
3. In order to elucidate the possible mechanisms involved in the secretion of staphylocoagulase, the influence of cerulenin, quinacrine and protein synthesis inhibitors on the production of staphylocoagulase in magnesium- and oxygen-limited chemostat cultures of S. aureus 104 was investigated (Chapter 4).

4. During prolonged growth of S. aureus strain 104 and strain NCTC 8178 in continuous culture, staphylocoagulase-negative mutants accumulated in the chemostat. The characteristics of these mutant strains and their competitive ability with respect to their parent strains in chemostat cultures under different growth conditions were established.
Furthermore these staphylocoagulase-negative strains were used to elucidate the regulation of the synthesis of staphylocoagulase and of exoproteins in general.
5. Since the ability to produce staphylocoagulase is a generally accepted criterion for the identification of S. aureus, the availability of a reliable assay method of staphylocoagulase is indispensable. Therefore, a rapid and direct assay of staphylocoagulase for the routine identification of S. aureus using the chromogenic substrate Chromozym TH was developed (Chapter 6).

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Influence of Cultivation Conditions on the Production of Staphylocoagulase by *Staphylococcus aureus* 104

High yields of staphylocoagulase from *Staphylococcus aureus* 104 were obtained in a simple salts medium supplemented with glycerol, casein hydrolysate and three vitamins. Conditions of oxygen-limitation (dissolved oxygen concentration $\leq 2\%$), a pH of 7.4, a temperature of 35 °C and a 1 in 10 inoculum of overnight culture were required for optimal yields of staphylocoagulase.

INTRODUCTION

Staphylocoagulase is an extracellular protein produced by potentially pathogenic strains of *Staphylococcus aureus*. However, its precise role in the pathogenesis of infections due to *S. aureus* is not clear.

Several reports describe the partial purification of staphylocoagulase, but none of these preparations were of sufficient homogeneity to assess the mode of action of the enzyme (Blobel *et al.*, 1960; Duthie & Haughton, 1958; Jacherts, 1956; Jeljaszewicz, 1958; Murray & Ghodes, 1960; Siwecka & Jeljaszewicz, 1968; Soulier *et al.*, 1967; Stutzenberger & San Clemente, 1968; Tager, 1948; Zolli & San Clemente, 1963). Bas *et al.* (1974) found that purified staphylocoagulase was a single-chain protein with a molecular weight of 61 000, an isoelectric point at pH 4.53 and with aspartic acid as the N-terminal amino acid. Hemker *et al.* (1975) reported that the activation of human prothrombin by staphylocoagulase occurred stoichiometrically, one molecule of staphylocoagulase forming a complex with one of prothrombin without splitting the peptide bonds. Therefore the thrombin activity of this complex was probably not due to proteolytic breakdown of prothrombin to thrombin, but to a change of the tertiary structure of prothrombin that unmasked a thrombin-like active centre.

Various authors have described different conditions for optimal production of staphylocoagulase, presumably due to variations in strains, media and cultivation conditions. Therefore a new investigation was undertaken to assess the effects of medium composition and environmental factors on the formation of staphylocoagulase by *S. aureus* 104 when grown in batch cultures. This strain was chosen because high yields of enzyme had been obtained with it (Bas *et al.*, 1974; Soulier *et al.*, 1967).

METHODS

Bacterial strains. *Staphylococcus aureus* 104 (after Tager) was kindly provided by Dr J. P. Soulier (Centre National de Transfusion Sanguine, Paris). *Staphylococcus aureus* NCTC 8178 was from the National Collection of Type Cultures, London. Stock cultures were maintained on nutrient agar slants at 4 °C and were subcultured monthly.

Cultivation media. Brain Heart Infusion (Difco) contained 37 mg ml⁻¹. Yeast extract medium contained a simple salts mixture described by Evans *et al.* (1970) (omitting NH₄Cl) with a Mg²⁺ concentration of 100 µM,

Fig. 1. Relation between clotting time and activity of crude staphylocoagulase.

supplemented with (per ml) 1 mg glycerol and 20 mg yeast extract (Difco). Casein hydrolysate medium contained the simple salts mixture of Evans *et al.* (1970) (omitting NH_4Cl) with a Mg^{2+} concentration of $100 \mu\text{M}$, supplemented with (per ml) 20 mg Casamino acids (Difco), 1 mg glycerol and $20 \mu\text{l}$ vitamin solution. Synthetic medium contained the simple salts mixture of Evans *et al.* (1970) with a Mg^{2+} concentration of $100 \mu\text{M}$, supplemented with (per ml) 1 mg glycerol, $20 \mu\text{l}$ vitamin solution and the following amino acids (mg): L-Asp, 0.76; L-Thr, 0.64; L-Ser, 1.12; L-Glu, 2.28; L-Pro, 0.32; L-Gly, 0.32; L-Ala, 1.16; L-(Cys)₂, 0.16; L-Val, 1.04; L-Met, 0.32; L-Ile, 0.84; L-Leu, 1.32; L-Tyr, 0.44; L-Phe, 1.36; L-Lys, 1.66; L-His, 0.28; L-Try, 0.20; L-Arg, 0.68. The vitamin solution contained (per ml double glass-distilled water) $300 \mu\text{g}$ thiamin, $100 \mu\text{g}$ nicotinic acid and 30 ng biotin. This mixture was heat sterilized separately at pH 4.5. The pH of all media was adjusted to 7.4.

Cultivation methods. *Staphylococcus aureus* 104 was cultivated in 20 ml medium in 100 ml Erlenmeyer flasks at 35°C . Some cultures were shaken in a gyrotary waterbath (G-76, New Brunswick) at $190 \text{ rev. min}^{-1}$ and a displacement of 13 mm. The aeration efficiency in the shaken flasks, as determined by the sulphite oxidation method (Cooper *et al.*, 1944), was less than $5 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$.

The organism was also cultivated in stirred fermenters (Bioflo C30, New Brunswick), with a culture volume of 300 to 400 ml, depending on the stirrer speed. No baffles were used. Air was flushed over the culture surface at a rate of 300 ml min^{-1} . The aeration efficiency, determined by the sulphite oxidation method (Cooper *et al.*, 1944), was less than $5 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$ at stirrer speeds lower than $400 \text{ rev. min}^{-1}$. The dissolved oxygen concentration was less than 2% at speeds lower than $400 \text{ rev. min}^{-1}$. The pH was maintained constant at 7.4 by automatic addition of 2 M-KOH. The temperature was held at 35°C .

Staphylocoagulase assay. The staphylocoagulase activity was determined on the basis of the clotting time obtained when 0.1 ml culture supernatant (1 ml culture was centrifuged at $12500 g$ for 2 min) was mixed with 0.1 ml rabbit plasma and incubated at 37°C (Soulie *et al.*, 1967). The amount of staphylocoagulase was calculated from a reference curve constructed from clotting times obtained with dilutions (in Michaelis buffer) of a pool of 10 crude staphylocoagulase preparations. This pool was considered to contain 1000 arbitrary units (U) ml^{-1} (Fig. 1).

Preparation of crude staphylocoagulase. Crude staphylocoagulase was prepared by a modification of the method described by Soulie *et al.* (1967). *Staphylococcus aureus* 104 was cultivated in 40 ml casein hydrolysate medium in 200 ml Erlenmeyer flasks at 35°C without shaking. After 24 h the cultures were centrifuged for 30 min at $15380 g$; the supernatant was brought to pH 3.5 with 3 M-HCl and left for 18 h at 0°C . The precipitate was collected by centrifugation for 30 min at $7000 g$, washed once with 0.15 M-sodium acetate buffer (pH 3.8), dissolved in 0.15 M-NaCl and brought to pH 7.4 with NaOH.

Staphylocoagulase production is defined here as net production, i.e. the enzyme synthesized by some or all of the bacterial cells plus its release from the living or dead cells minus any inhibition or destruction outside the cell.

RESULTS AND DISCUSSION

To simplify the procedure for purification of staphylocoagulase produced by *S. aureus* 104, it was necessary to replace the Brain Heart Infusion medium that is generally used by a more defined medium. The major disadvantage of Brain Heart Infusion is its high lipid content that necessitates a very time-consuming lipid-extraction step in the purification procedure (Bas *et al.*, 1974).

The amounts of staphylocoagulase produced in various media are shown in Table I. The

Table 1. *Staphylocoagulase production by Staphylococcus aureus* 104 in various media

Erlenmeyer flasks (100 ml) containing 20 ml medium were inoculated with 2 ml overnight culture and cultivated at 35 °C without shaking or with shaking at 190 rev. min⁻¹. After 18 h, staphylocoagulase and absorbance at 540 nm (figures in parentheses) were determined.

Medium	Staphylocoagulase (U ml ⁻¹)	
	Static culture	Shaken culture
Brain Heart Infusion	13 (6.29)	24 (11.69)
Yeast extract medium	21 (4.24)	7 (11.75)
Casein hydrolysate medium	65 (4.73)	17 (9.53)
Casein hydrolysate (vitamin-free) medium	10 (5.05)	< 1 (8.77)
Synthetic medium	42 (6.53)	5 (9.68)

shaken cultures, except those in Brain Heart Infusion, produced less staphylocoagulase than did the static cultures. Several authors (Duthie & Haughton, 1958; Fahlberg & Marston, 1960; Stutzenberger & San Clemente, 1968; Tager, 1948) have reported that agitation is essential for the production of large amounts of staphylocoagulase. Soulier *et al.* (1967) found that static cultures of *S. aureus* 104 in Brain Heart Infusion produced 15 U ml⁻¹ (1 l Roux bottles containing 200 ml medium).

The reason for the diminished staphylocoagulase production obtained with cultures grown in the casein hydrolysate medium based on vitamin-free Casamino acids rather than Casamino acids is obscure. Addition of different mixtures of vitamins (up to 12) gave no increase in staphylocoagulase production. Further, investigations regarding the composition and preparation of the Difco products used failed to reveal the cause of the difference in yields.

A synthetic medium for *S. aureus* 104 has not been described previously, although Marston & Fahlberg (1960) described a synthetic medium suitable for *S. aureus* NCTC 8178. In our hands, neither *S. aureus* 104 nor NCTC 8178 produced any staphylocoagulase in this medium (data not shown). However, with the synthetic medium described here, both *S. aureus* 104 and NCTC 8178 (the latter grown with agitation) produced considerable amounts of staphylocoagulase (65 and 40 U ml⁻¹, respectively).

Staphylocoagulase was produced optimally (120 U ml⁻¹) in medium containing 5% (w/v) casein hydrolysate. The Mg²⁺ concentration was critical for staphylocoagulase production, being optimal at 50 to 100 µM; higher concentrations were inhibitory. Glycerol (and glucose) concentrations higher than 0.2% (w/v) also inhibited staphylocoagulase production. Addition of bovine serum albumin (up to 1% w/v), previously considered necessary to obtain high staphylocoagulase yields (Altenbern, 1966), failed to give any enhancement. For staphylocoagulase purification, the casein hydrolysate medium was chosen for bulk production. For physiological studies (particularly continuous culture studies), the synthetic medium and the casein hydrolysate medium seemed the most promising since both media are free of lipid and protein. Removal of any of the amino acids from the synthetic medium reduced the amount of staphylocoagulase produced.

In all the media listed in Table 1, staphylocoagulase production occurred only in the exponential growth phase. Optimal amounts of enzyme were obtained with an initial pH of 7.4, an incubation temperature of 35 °C and a 1 in 10 inoculum of overnight culture.

The complex nature of the regulation of staphylocoagulase production is illustrated by Fig. 2. In agitated yeast extract medium, production of staphylocoagulase by *S. aureus* 104 reached a maximum after 1.5 h and then declined rapidly. If shaking was stopped 1.5 h after inoculation, the staphylocoagulase level still declined, but after several hours synthesis restarted. When the experiment shown in Fig. 2 was repeated in stirred fermenters, the decrease in staphylocoagulase level (from 18 to 4 U ml⁻¹) at 300 rev. min⁻¹ was accompanied by a decrease in pH (to pH 5.5). When growth continued, the pH rose to 8 to 8.5 and staphylo-

Fig. 2. Staphylocoagulase production by *Staphylococcus aureus* 104 as a function of the cultivation time. Four Erlenmeyer flasks (100 ml), each containing 20 ml yeast extract medium, were inoculated with 2 ml overnight culture and incubated at 35 °C and 190 rev. min⁻¹. After 1.5 h, as marked by the arrow, two flasks were transferred to a non-shaking waterbath at 35 °C and the experiment was continued. ---, Static cultures; —, shaken cultures; ●, staphylocoagulase; ○, absorbance at 540 nm.

Table 2. *Staphylocoagulase production by Staphylococcus aureus* 104

Yeast extract medium (20 ml) was inoculated with 2 ml overnight culture and shaken at 190 rev. min⁻¹ at 35 °C. After 1.5 h, the culture was centrifuged at 5000 g for 15 min. The supernatant contained 27 U staphylocoagulase ml⁻¹. Sediment and supernatant were then incubated in various mixtures (as indicated) and staphylocoagulase production in these mixtures was determined after a further 1.5 h.

Incubation mixture	Staphylocoagulase production (U ml ⁻¹)
Control; culture not centrifuged	— 16
Sediment + 20 ml supernatant	— 16
Sediment + 20 ml fresh yeast extract medium	0.5
2 ml Overnight culture + 18 ml supernatant	17

coagulase production began again, attaining 15 U ml⁻¹ after overnight growth. Under conditions in which the pH was maintained at 7.4, there was little reduction in staphylocoagulase levels (8 U ml⁻¹ at 200 rev. min⁻¹, remaining constant during 18 h cultivation; 18 U ml⁻¹ at 300 rev. min⁻¹, decreasing during 18 h cultivation to 16 U ml⁻¹). Similar observations were made by Duthie & Haughton (1958) for *S. aureus* NCTC 8178 growing in a casein hydrolysate medium. They found that the amount of staphylocoagulase reached a maximum 80 min after inoculation, and then decreased rapidly. The maximum level of staphylocoagulase under the conditions of Fig. 2 was strongly dependent on the inoculum size. Optimal staphylocoagulase production (42 U ml⁻¹) occurred with a 1 in 10 inoculum.

The experiment shown in Table 2 indicated that the reduction in staphylocoagulase production is probably not due to exhaustion of medium constituents or production of an inhibitory substance. Moreover, regardless of the growth phase, proteolytic activity was never demonstrated in the culture supernatants.

Similar findings were obtained when *S. aureus* 104 was subcultured in shaken flasks

Fig. 3. Staphylocoagulase production by *Staphylococcus aureus* 104 as a function of the cultivation time. Thirty-two Erlenmeyer flasks (100 ml), each containing 20 ml casein hydrolysate medium, were inoculated with 2 ml overnight culture and incubated at 35 °C without agitation. Every hour, absorbance at 540 nm (○), staphylocoagulase (●) and pH (■) were determined in two flasks.

Fig. 4. Influence of the ratio culture volume/flask volume on staphylocoagulase production by *Staphylococcus aureus* 104. Erlenmeyer flasks (100 ml) containing various volumes of casein hydrolysate medium and a 1 in 10 inoculum of overnight culture were incubated at 35 °C without agitation. After 18 h, absorbance at 540 nm (○) and staphylocoagulase (●) were determined.

containing casein hydrolysate or yeast extract medium. After two subcultures, production of staphylocoagulase ceased completely and remained at zero during 14 further subcultures; but when the culture was subcultured to casein hydrolysate or yeast extract medium and incubated statically, staphylocoagulase production began again and regained its full potential after 2 to 3 subcultures. When *S. aureus* 104 was subcultured up to 14 times in casein hydrolysate or yeast extract medium incubated statically, production of staphylocoagulase remained high. The loss of ability of staphylococci to produce staphylocoagulase, described by Tager (1974), is evidently dependent on the cultivation conditions.

The production of staphylocoagulase in casein hydrolysate medium at different growth phases is shown in Fig. 3. After a prolonged lag phase, rapid growth was accompanied by production of the enzyme. When growth ceased, there was no further change in staphylocoagulase level. The pH remained practically constant, which is an important factor in optimal staphylocoagulase production.

That agitation and inoculum size influence staphylocoagulase production so markedly suggests that aeration efficiency is important. Thus staphylocoagulase production takes place only when the aeration efficiency is less than a critical value, as indicated by the experiment shown in Fig. 4. The maximum production is related to the final culture density, because at a medium volume/flask volume ratio higher than 0.2, both production of staphylocoagulase and cell density decrease, indicating that the amount of staphylocoagulase produced per cell is approximately constant.

Oxygen measurements made with a Clark electrode during growth of *S. aureus* 104 in casein hydrolysate medium without agitation revealed that in the lag phase the dissolved oxygen concentration was less than 2%. Redox potential measurements (which also give an indication of the oxygen concentration) with a platinum and a calomel electrode indicated that the redox potential of the culture decreased rapidly during the first 2 h cultivation, from +400 mV to +100 mV, and then continued to decrease steadily for the next 21 h to -80 mV. Staphylocoagulase was produced at redox potentials between +40 and +60 mV, i.e. during a period of considerable oxygen-limitation. For other extracellular enzymes (phosphatase, staphylokinase, enterotoxin A, nuclease) oxygen-limiting conditions are also necessary for high amounts of enzyme to be formed (Arvidson *et al.*, 1976; Carpenter & Silverman, 1976).

Although it is apparent that oxygen plays a major role in staphylocoagulase production by *S. aureus* 104, other factors are involved, such as pH, growth rate and nutrient-limitation, which need to be studied further. Experiments concerning the influence of these factors might usefully employ continuous culture techniques. However, the possibility remains that staphylocoagulase production by *S. aureus* 104 is a transient-state and not a steady-state phenomenon, so that continuous culture may not be applicable.

It is not clear whether staphylocoagulase is a true exo-enzyme. Indeed only low levels of the enzyme can be demonstrated in cell lysates of *S. aureus* 104 [prepared with lysostaphin, see also Altenbern (1966)], but the possibility that a high percentage of lysis occurs during growth cannot be excluded. Extracellular RNA and DNA, released during lysis, cannot be demonstrated because *S. aureus* 104 also produces high levels of a nuclease which breaks down RNA and DNA. Moreover, Blobel & Berman (1960) demonstrated that staphylocoagulase is also located on the cell surface. Therefore growth conditions that result in unstable walls might be favourable for high levels of staphylocoagulase. The non-specific stimulatory effect of serum albumin as described by Altenbern (1966), and the influence of the Mg^{2+} concentration on staphylocoagulase production, indicate that the wall composition may well play an important role.

We wish to thank Dr O. N. Neijssel for helpful discussions and Professor D. W. Tempest for critically reading the manuscript.

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Continuous culture studies on the production of staphylocoagulase by *Staphylococcus aureus*

ENGELS, W., KAMPS, M. A. F. and VAN BOVEN, C. P. A. 1980. Continuous culture studies on the production of staphylocoagulase by *Staphylococcus aureus*. *Antonie van Leeuwenhoek* **46**: 533-550.

The production of staphylocoagulase was studied with continuous cultures of various *S. aureus* strains in a simple salts medium supplemented with mannitol, casein hydrolysate and three vitamins. Conditions of low oxygen availability and magnesium-limitation were required for optimal steady-state staphylocoagulase production. It was demonstrated that the specific rate of staphylocoagulase production was dependent on the growth rate.

In two bovine strains, the production rate pattern was similar to that of an inducible enzyme sensitive to catabolite repression, although no specific inducer or repressor could be demonstrated. The human strain, on the other hand, produced staphylocoagulase constitutively. In all strains the specific rate of production of total extracellular protein was strictly proportional to the growth rate. The bovine strains produced 6 times more staphylocoagulase in chemostat culture as compared with batch cultures of the same organisms.

It is likely that mannitol functioned as an energy source rather than as a carbon source because it was converted for a major part to acetate and for a minor part to lactate and not to new cell material. Repression of staphylocoagulase production by mannitol, acetate or lactate was not observed. The probable nature of the regulating mechanism(s) underlying staphylocoagulase production is discussed.

INTRODUCTION

The production of the various exoenzymes and exotoxins by *Staphylococcus aureus* has been investigated extensively in batch cultures, but applications of continuous culture techniques in these studies have not been widespread (Arvidson, Holme and Wadström, 1970; Arvidson et al., 1976; Holme and Arvidson, 1976; Holme and Wadström, 1971; Jarvis, Laurence and Pritchard, 1975). In particular the influence of cultivation conditions on the production of staphylo-

coagulase by *S. aureus* in chemostat cultures has, as far as we know, not been studied before.

It was previously shown (Engels, Kamps and van Boven, 1978) that *S. aureus* strain 104, in batch cultures, produced optimal amounts of staphylocoagulase under oxygen-limited growth conditions. Moreover, it was demonstrated that the concentrations of the sources of magnesium and carbon were also critically important. Therefore, a study was undertaken to investigate the effect of these parameters and of the growth rate on the production of staphylocoagulase in *S. aureus* strain 104 and two bovine wild type strains under steady-state growth conditions.

In this way more information about the role of staphylocoagulase in pathogenesis might be obtained because also in its host, *S. aureus* will grow with sub-maximal growth rates and under substrate-limited growth conditions.

The results of these studies are reported in the present paper.

MATERIALS AND METHODS

Organisms

Staphylococcus aureus strains (for characteristics see Table 1) were maintained

Table 1. Characteristics of the *Staphylococcus aureus* strains.

Source	Strain		
	20, bovine ¹	15, bovine ¹	104, human ²
Staphylocoagulase reaction with:			
human plasma	+	+	+
rabbit plasma	+	+	+
bovine plasma	+	—	—
DNase	+	+	+
Protease	—	—	—
Phosphatase	+	+	+
Tween 80 hydrolysis	—	—	+
Haemolysins	+	+	+
Staphylokinase	—	—	+
Penicillinase	+	+	—
Crystal violet reaction	A/B	B	C/D
Phage type	I, III, bovine set	I, III, bovine set	III
Pigment	yellow	yellow	yellow

¹ These strains had been selected from 261 *S. aureus* strains provided by Dr J. M. F. Seas (Animal Health Centre, Heythuizen, The Netherlands).

² *S. aureus* 104 was kindly provided by Dr J. P. Soulier (Centre National de Transfusion Sanguine, Paris).

on nutrient agar slopes by monthly subculture. *S. aureus* strain 20 and 15 were characterized as bovine types on the basis of phage type and crystal violet reaction (Szulga et al., 1976). However, the staphylocoagulase activity of strain 20 with human and rabbit plasma was five to ten times higher than with bovine plasma, whereas staphylocoagulase produced by strain 15, did not react with bovine plasma at all.

Growth conditions

Organisms were cultivated in Bioflo C30 chemostats (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) in a casein hydrolysate medium containing a simple salts mixture described by Evans, Herbert and Tempest (1970) (omitting NH_4Cl) with a Mg^{2+} concentration of 100 μM , supplemented with (per liter) 10 g Casamino acids (Difco), 2 g mannitol, 6 mg thiamin, 2 mg nicotinic acid and 0.6 μg biotin. The culture volume was 300–350 ml, depending on the stirrer speed. The pH was maintained at a value of 7.4 by means of an automatic titrator with 2 M KOH as the titrant. The temperature was held at 30 °C.

To minimize excessive foaming the baffles had to be removed, the impeller speed kept at moderate values and air flushed over the surface (300 ml/min). Nevertheless, an antifoaming agent was still necessary and was added automatically at regular time intervals. The AF-antifoam (purchased from Dow Corning), added as a 2% (w/v) solution, suppressed effectively foam formation by the bovine strains; in case of strain 104, such high dosages of this antifoam had to be added that staphylocoagulase production was strongly inhibited (see also Jarvis et al., 1973). The same phenomenon was observed with polypropylene glycol P2000. Therefore, another antifoaming agent on silicone base (purchased from BDH) was used, which had a high effectivity and could be added at a non-inhibitory concentration of 0.5% (w/v).

Analytical methods

Bacterial dry weight was measured by the procedure of Herbert, Phipps and Strange (1971).

Total extracellular protein was determined, after overnight dialysis of culture supernatant against double glass distilled water at 0–4 °C, according to Lowry et al. (1951).

Dissolved oxygen concentrations (D.O.C.) were measured with a Clark oxygen probe (type YSI 4004, Yellow Spring Instrument Co., Yellow Springs, U.S.A.). The electrode was sterilized with ethanol–formaldehyde (1:1) vapour. In all experiments the D.O.C. measured in this way was 0–1% except when indicated otherwise.

Mannitol was determined according to Dawes, McGill and Midley (1971).

Acetate was assayed enzymically by the method of Holz and Bergmeyer (1974).

L-Lactate was assayed with L-Lactate dehydrogenase by the method of Gutmann and Wahlefeld (1974).

D-Lactate was assayed with D-Lactate dehydrogenase by the method of Gawehn and Bergmeyer (1974).

Acetoin was determined according to Westerfeld (1945).

Magnesium was measured with an atomic absorption spectrophotometer, Perkin-Elmer type 305B. In all experiments the Mg^{2+} concentrations in the culture fluid were in the range of 5–7 μM except when indicated otherwise.

Amino acid composition of culture supernatants and medium was analyzed with an automatic amino acid analyser (Locarte, London, U.K.).

Gas analysis. Oxygen consumed, and carbon dioxide produced by the cultures were determined by passing the dried effluent gas from the fermentor through an oxygen analyser (Taylor Servomex Type 0A 272, Crowborough, Sussex, England) and a carbon dioxide analyser (model IRGA 10, Grubb Parsons & Co. Ltd., Walkergate, Newcastle upon Tyne, England). Prior to each determination, the analysers were calibrated with a standard gas mixture obtained from Hoek Loos (Amsterdam, The Netherlands).

P.P.S.B., (Prothrombin, Proconvertin, Stuart-Prower factor, antihæmophilic factor B), a concentrate of the blood clotting factors II, VII, X and IX was prepared according to Soulier et al. (1969) and kindly provided by Miss A. D. Muller, Dept. of Biochemistry, State University of Limburg.

Staphylocoagulase. A more direct and quantitative spectrophotometric assay was developed because in the "classic" clotting assay false positive or negative results can be obtained due to other extracellular products present in cultures of *S. aureus* (Wegrzynowicz et al., 1979). Moreover, clotting times are very difficult to standardize. Staphylocoagulase activity was assayed by a modification of the method described by Kirchhof, Vermeer and Hemker (1978) originally used for the determination of thrombin activity. The reaction mixture (600 μl) contained 20–100 μl culture supernatant, 72 mM triethanolamine buffer pH 8.4, 144 mM NaCl, 166 μM Chromozym TH (Boehringer, Mannheim) and 0.002 ml human P.P.S.B. solution, containing 1.9 mg prothrombin/ml, as the prothrombin source. One unit was defined as the amount of enzyme giving an increase in absorbance at 405 nm of 1.0 per min at 37 °C. Because proteolytic activity was not detected in absence of prothrombin and no proteolytic enzymes in the culture supernatants could be demonstrated, it can be concluded that staphylocoagulase activity was measured specifically. Staphylocoagulase production by *S. aureus* strains was also screened on agar plates containing 52 mg/ml Brain Heart Infusion agar (Difco) and 10% (v/v) human plasma. Staphylocoagulase producing colonies were surrounded by haloes of 4–7 mm in diameter after 18 h of cultivation at 37 °C.

Penicillinase was assayed by the method of Sargent (1968).

DNase was assayed according to Heins, Taniuchi and Anfinsen (1966).

Phosphatase was assayed according to Ghosh, Wouters and Lampen (1971).

To a reaction mixture (1 ml) containing 50 mM Tris-HCl pH 7.4, 0.5 mM $MgCl_2$ and 5.5 mM sodium-*p*-nitrophenylphosphate, 0.1 ml culture supernatant was added. After 30 min of incubation at 37°C, the reaction was terminated by adding 10 ml 0.02 M NaOH. One unit was defined as the amount of enzyme giving an increase in absorbance at 405 nm of 5.

Protease was determined with the Azocoll method (Calbiochem) according to Aronson, Angelo and Holt (1971). The absorbance was measured at 520 nm.

L-Lactate dehydrogenase and D-Lactate dehydrogenase were assayed according to Bergmeyer and Bernt (1974) with L-lactate and D-lactate as substrate, respectively.

Haemolysis, Crystal violet-test, Tween 80-hydrolysis, Staphylokinase, and phage typing were carried out by the National Institute of Public Health (R.I.V., Bilthoven, The Netherlands) according to Meyer (1967).

Lysozyme was assayed according to Shugar (1952).

RESULTS

Staphylocoagulase production by S. aureus strain 20

Chemostat cultures of *S. aureus* strain 20 were set up and the effect of various environmental parameters on the production of staphylocoagulase by this strain was studied. The medium was composed so as to provide a magnesium limitation and the aeration efficiency of the culture vessel was diminished to limit deliberately the availability of oxygen.

Fig. 1. Influence of dilution rate on the specific rates of production of staphylocoagulase and total extracellular protein, and on the production of biomass in a chemostat culture of *S. aureus* strain 20 (400 rpm, 30 °C).

○ Bacterial dry weight; ● staphylocoagulase; △ total extracellular protein (TEP); ▲ ratio staphylocoagulase/total extracellular protein; □ mannitol concentration in the culture fluid.

Table 2. Influence of the mannitol concentration on the production of staphylocoagulase in a chemostat culture of *Staphylococcus aureus* strain 20. ($D = 0.24 \pm 0.01 \text{ h}^{-1}$, 400 rpm, 30 °C).

	Mannitol concentration in the medium reservoir (mM)			
	0	11 ¹	22	44
Bacterial dry weight (mg/ml)	1.64	2.00	2.08	2.17
Mannitol in culture fluid (mM)	0	0.66	6.6	28.6
Staphylocoagulase (U/mg dry wt)	0.19	0.64	0.63	0.61
Total extracellular protein ($\mu\text{g}/\text{mg}$ dry wt)	57	65	84	76
L-Lactate (mM)	0.15	0.20	0.22	0.22
Acetate (mM)	0	20.0	26.5	21.7

¹ Routinely a concentration of 11 mM mannitol is used (see Materials and Methods).

The first series of experiments were performed to study the effect of the dilution rate of the culture on the production of staphylocoagulase. Fig. 1 shows that the specific rate of production of this protein increased with the dilution rate up to $D = 0.28 \text{ h}^{-1}$ ($\mu_{\text{max}} = 0.8 \text{ h}^{-1}$); a further increase of the dilution rate resulted in a substantially lower specific production rate. The production of staphylocoagulase did not follow the pattern of extracellular protein production: at a dilution rate of 0.33 h^{-1} the ratio staphylocoagulase/total extracellular protein was lower by a factor of 3 as compared with those of the lower dilution rates. Nevertheless, the amount of staphylocoagulase produced in chemostat culture by this strain was approximately six times higher than that generally found in batch cultures of this organism (0.6 U/mg vs. 0.1 U/mg).

It has to be mentioned that steady-states were attained relatively slowly. It took at least 8–10 changes of culture volume before a new steady-state was established. This phenomenon is most likely related to the nature of the growth limitation: in a number of cases it has been observed by other authors (Kurowski, Fensom and Pirt, 1973; Neijssel and Tempest, 1979) that magnesium-limited chemostat cultures were very difficult to maintain at a steady-state.

It was of interest to determine whether staphylocoagulase production was due to cell-lysis. Therefore, we assayed the culture fluid for the presence of intracellular enzymes. L- and D-lactate dehydrogenase could never be demonstrated, whereas high intracellular levels of these enzymes were present (860 and 440 mmoles/min·mg protein, respectively, at $D = 0.28 \text{ h}^{-1}$). Lysozyme also could not be detected in the culture fluid, whereas high levels were found in the culture fluid of shaken-batch cultures (see also Arvidson et al., 1970). Therefore cell-lysis must have been minimal.

From Table 2, it can be concluded that mannitol is necessary for optimal staphylocoagulase and biomass production, but that there is no catabolite repres-

sion at higher concentrations. In the absence of mannitol the production of staphylocoagulase decreased considerably, whereas no acetate is produced. It can be calculated that at mannitol concentrations of 11, 22 and 44 mM, mannitol is converted to acetate for 100, 87 and 70 %, respectively. Since the accumulation of the acetate is also associated with increased staphylocoagulase production, the effect of addition of 10 mM acetate in the absence of mannitol was investigated. The major part of acetate was consumed (8 mM); however, no increase of the staphylocoagulase and biomass production was observed (data not shown).

Another factor influencing staphylocoagulase production in batch cultures was the availability of oxygen (Engels et al., 1978). Because the baffles were removed from the fermentor vessel, the aeration efficiency could be influenced

Fig. 2. Influence of the stirrer speed on the production of staphylocoagulase, total extracellular protein, biomass, L-lactate and acetate in a chemostat culture of *S. aureus* strain 20 ($D = 0.25 \pm 0.01 \text{ h}^{-1}$, 30°C).

○ Bacterial dry weight; ● staphylocoagulase; △ total extracellular protein (TEP); ▲ ratio staphylocoagulase/total extracellular protein; □ L-lactate; ■ acetate.

by varying the stirrer speed. The influence of the stirrer speed on the staphylocoagulase production is shown in Fig. 2. The dissolved oxygen concentrations at 300 and 400 rpm were 0–1 % and 1–2 %, respectively. At higher speeds the dissolved oxygen concentrations increased to 21, 45 and 82 % at 500, 600 and 700 rpm, respectively. At a stirrer speed of 300 rpm, staphylocoagulase, biomass and protein production were low, while L-lactate was the major fermentation product. Besides 10.5 mM L-lactate also 6 mM D-lactate and 5.5 mM acetate was formed. At higher stirrer speeds, staphylocoagulase production increased up to an optimum at 600 rpm. At 700 rpm staphylocoagulase and total extracellular protein production were considerably inhibited. L-Lactate production decreased with increasing stirrer speeds and acetate became the main fermentation product. The decline in acetate production at higher stirrer speeds is most probably due to a metabolic change from oxygen limitation to oxygen excess, resulting in a more complete oxidation of mannitol.

It has to be noted that anaerobic growth in this medium is not possible. Supplying the culture with nitrogen gas instead of air gave an immediate and complete inhibition of growth and staphylocoagulase production, resulting in a wash-out of the *S. aureus* culture.

At 300 and 400 rpm a complete carbon balance with respect to mannitol could be obtained: the amount of mannitol consumed was equal to the total amount of acetate, L- and D-lactate produced. Besides acetate and lactate, acetoin was found in concentrations of 50–100 μM . Because these concentrations of acetoin were low and mostly only detectable during the transient state when the culture was changed from one steady-state to another, acetoin was not taken into account in the carbon balance.

Oxygen consumption measurements at 400 rpm indicated that the q_{O_2} was 4.3 mmoles per gram dry weight and per hour. This is in agreement with a complete conversion of mannitol to acetate and carbon dioxide since this reaction would give a theoretical q_{O_2} of 4.1 mmoles per gram dry weight and per hour.

Although steady-state conditions at a stirrer speed of 600 rpm gave an optimal staphylocoagulase production, these conditions were difficult to maintain because they often gave sudden excessive foaming which inactivated staphylocoagulase and inhibited its production. The latter is caused by a dramatic decrease in aeration efficiency due to impaired mixing and a wash-out of cells. Therefore, a stirrer speed of 400 rpm was used in the other experiments.

Stimulation of staphylocoagulase production by enrichment of the air influx with carbon dioxide, as reported by Tirunarayanan (1966) was not observed. On the contrary, concentrations higher than 5 % (v/v) were found to be strongly inhibitory under our growth conditions. In this connection, it has to be noted that when the air was not enriched with carbon dioxide, the concentration of carbon dioxide in the gasphase at a dilution rate of 0.25 h^{-1} and a stirrer speed of 400 rpm was already approximately 1 % (v/v).

It has been proposed by Hueting and Tempest (1977) that high concentrations

of acetate and other low-molecular fatty acids exert a strong influence on the metabolism of microorganisms, most probably due to an uncoupling of oxidative phosphorylation. Since *S. aureus* produced acetate and lactate from mannitol, we investigated the effect of an extra addition of 20 mM acetate and L-lactate to the growth medium on the production of staphylocoagulase. It was found that these acids did not inhibit staphylocoagulase production and that L-lactate was mainly converted into acetate (Table 3).

We reported previously (Engels et al., 1978) that high concentrations of magnesium inhibited staphylocoagulase production in batch cultures. In Table 4 the influence of the magnesium concentration on staphylocoagulase and total extracellular protein production is shown. Magnesium at 400 μM completely inhibited staphylocoagulase production while protein production decreased with 50%. No significant effect was found on bacterial dry weight.

The influence of the casein hydrolysate concentration on staphylocoagulase production is shown in Table 5. It can be seen that staphylocoagulase production decreased only slightly with increased casein hydrolysate concentrations whereas the total extracellular protein production was inhibited considerably. An increase of the casein hydrolysate concentration from the normally used 10 mg/ml also did increase the acetate concentration in the culture fluid. It is clear from this experiment that at casein hydrolysate concentrations of 20 and 40 mg/ml acetate was not only formed from mannitol but also from amino acids.

Table 3. Influence of the acetate and lactate concentration on the production of staphylocoagulase and metabolism in a chemostat culture of *Staphylococcus aureus* strain 20. ($D = 0.24 \pm 0.01 \text{ h}^{-1}$, 400 rpm, 30°C).

Addition	Acetate (mM)	L-Lactate (mM)	Staphylo- coagulase (U/mg dry wt)	Bacterial dry wt (mg/ml)
None	15.6	0.16	0.56	2.20
20 mM Acetate	32.0	0.16	0.53	2.22
20 mM L-Lactate	31.7	0.22	0.54	2.37

Table 4. Influence of the magnesium concentration on the production of staphylocoagulase in a chemostat culture of *Staphylococcus aureus* strain 20. ($D = 0.25 \pm 0.01 \text{ h}^{-1}$, 400 rpm, 30°C).

Mg ²⁺ medium reservoir (μM)	Mg ²⁺ culture fluid (μM)	Bact. dry wt (mg/ml)	Staphylo- coagulase (U/mg dry wt)	Total extra- cellular protein ($\mu\text{g}/\text{mg dry wt}$)
105	4	1.59	0.56	84
236	76	1.62	0.18	60
390	225	1.82	< 0.05	44

Table 5. Influence of the casein hydrolysate concentration on the production of staphylocoagulase in a chemostat culture of *Staphylococcus aureus* strain 20. ($D = 0.24 \pm 0.01 \text{ h}^{-1}$, 400 rpm, 30 °C).

	Casein hydrolysate (mg/ml)		
	10	20	40
Bacterial dry weight (mg/ml)	2.11	2.19	2.32
Staphylocoagulase (U/mg dry wt)	0.55	0.51	0.51
Total extracellular protein ($\mu\text{g}/\text{mg dry wt}$)	69	59	52
Acetate (mM)	20.0	26.5	32.9
L-Lactate (mM)	0.16	0.17	0.22
Mannitol (mM)	0.27	0.33	0.77
Osmolarity (mOsm)	185	324	565

Analysis of the amino acid composition of the culture fluids (data not shown) revealed that, when the casein hydrolysate concentration was 10 mg/ml, most amino acids were present in excess of growth requirement, except aspartate, threonine, serine, glutamate, glycine and alanine, which were almost completely consumed. When the casein hydrolysate concentration was raised to 20 mg/ml, only threonine and serine could not be detected in the culture fluid. At a concentration of 40 mg/ml casein hydrolysate, all amino acids were detectable in the extracellular fluid in concentrations greater than 1 mM. Cysteine and tryptophan were not present in the medium and tyrosine only in trace amounts. Addition of these amino acids did not affect staphylocoagulase or biomass production. An increase of the casein hydrolysate concentration also implicated an increase of the osmolarity of the medium due to the high NaCl content (14 % w/w) of casein hydrolysate. In Table 5, however, it can be seen that the slight changes in the production of staphylocoagulase and biomass did not correlate with the large changes in this parameter.

The influence of temperature on staphylocoagulase and protein production is shown in Fig. 3. Staphylocoagulase production was optimal at a temperature of 30–31 °C, while protein production increased with increasing temperatures. The biomass production is not influenced in this temperature range. It is of interest to note that the optimal temperature for staphylocoagulase production differed from the generally used cultivation temperature of 37 °C.

Production of staphylocoagulase and other extracellular enzymes by strain 15

To determine whether the kinetics of staphylocoagulase production was similar to that of the production of other extracellular enzymes, the excretion of penicillinase, DNase and phosphatase was also followed. However, the production of these enzymes by strain 20 as well as strain 104 was low in the casein hydrolysate medium and could not be determined reliably. Therefore, the influence of growth rate on the production of staphylocoagulase, penicillinase,

Fig. 3. Influence of temperature on the production of staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* strain 20 ($D = 0.25 \pm 0.01 \text{ h}^{-1}$; 400 rpm). ○ Bacterial dry weight; ● staphylocoagulase; △ total extracellular protein (TEP); ▲ ratio staphylocoagulase/total extracellular protein.

DNase and phosphatase was studied in strain 15, which produced the latter three enzymes in considerably higher amounts than the previously mentioned strains. In Table 6 it can be seen that the specific rate of production of extracellular enzymes was growth-rate dependent. The production pattern of staphylocoagulase and penicillinase had an optimum at a $D = 0.22 \text{ h}^{-1}$ ($\mu_{\max} = 0.8\text{--}1.0 \text{ h}^{-1}$); the production of DNase, phosphatase and total extracellular protein increased with increasing growth rates. The decrease in bacterial dry weight with increasing growth rates and a dissolved oxygen concentration lower than 1% correlates again with oxygen-limiting conditions. Also in this strain the specific rate of staphylocoagulase production in chemostat culture was approximately six times higher than that in batch culture (data not shown).

Table 6. Influence of dilution rate on some culture parameters and the production rate of extracellular enzymes in a chemostat culture of *Staphylococcus aureus* strain 15. (400 rpm, 30 °C).

	Dilution rate (h^{-1})			
	0.11	0.16	0.22	0.35
Bacterial dry weight (mg/ml)	1.45	1.35	1.28	0.94
Mannitol in culture fluid (mM)	0.11	0.22	0.66	7.2
Total protein ($\mu\text{g}/\text{mg dry wt} \cdot \text{h}$)	18.4	19.2	24.9	36.9
Staphylocoagulase (U/mg dry wt $\cdot$ h)	0.10	0.20	0.23	0.17
Penicillinase (mPerret U/mg dry wt $\cdot$ h)	0.13	0.27	0.55	0.19
DNase (U/mg dry wt $\cdot$ h)	0.05	0.10	0.24	0.38
Neutral phosphatase (m U/mg dry wt $\cdot$ h)	0.06	0.19	0.43	1.94

Staphylocoagulase production by strain 104

Because our study of staphylocoagulase production had been started in batch cultures of strain 104 (Engels et al., 1978) it was of interest to study the influence of the dilution rate on the staphylocoagulase production rate in a chemostat culture of strain 104.

The specific rate of staphylocoagulase production as depicted in Fig. 4, in-

Fig. 4. Influence of dilution rate on the specific rates of the production of staphylocoagulase and total extracellular protein, and on the production of biomass, L-lactate and acetate in a chemostat culture of *S. aureus* strain 104 (400 rpm, 30 °C).

○ Bacterial dry weight; ● staphylocoagulase; △ total extracellular protein (TEP); ▲ L-lactate; □ mannitol; ■ acetate.

creased to an optimum at $D = 0.76 \text{ h}^{-1}$. In contrast to the other strains used in this study, strain 104 produced less staphylocoagulase in chemostat culture than in batch culture (0.67 U/mg vs. 1.5–2.0 U/mg). At even higher dilution rates the production rate declined due to wash-out conditions ($\mu_{\text{max}} = 0.9\text{--}1.1 \text{ h}^{-1}$). The total extracellular protein production increased with increasing dilution rates. Bacterial dry weight decreased with increasing dilution rates indicating oxygen-limiting conditions.

Dissolved oxygen concentration measurements revealed values lower than 1% up to a $D = 0.36 \text{ h}^{-1}$. At higher dilution rates the dissolved oxygen concentration progressively increased to 41% at $D = 0.76 \text{ h}^{-1}$.

At dilution rates in the range of $0.20\text{--}0.36 \text{ h}^{-1}$ mannitol was mainly converted to acetate; at higher dilution rates also a minor amount of L-lactate was formed, while acetate production declined, a phenomenon similar to that observed in strain 20. No explanation can be given for the production of L-lactate under these conditions. Addition of 20 mM acetate at a $D = 0.76 \text{ h}^{-1}$ did not influence the specific rate of staphylocoagulase production.

Under conditions of optimal production of staphylocoagulase, staphylocoagulase-negative mutants of strain 104 invariably appeared after a number of generations and these mutants took over the staphylocoagulase-producing population for more than 90%. The same phenomenon was observed with strain NCTC 8178 (unpublished results). Because the precise moment of this take-over was mostly unpredictable, no further experiments were done with strain 104. In a separate study the selective advantages of the staphylocoagulase-negative mutants of *S. aureus* 104 and NCTC 8178 will be investigated.

DISCUSSION

In general, the production of extracellular enzymes follows one of four patterns and it is assumed that the relationship between enzyme production and growth rate depends on the characteristics of the regulating mechanisms involved (Bull, 1972; Dean, 1972; Glenn, 1976). The four patterns of enzyme production, as reviewed by Wiersma and Harder (1978), includes constitutive enzymes, constitutive enzymes controlled by catabolite repression, inducible enzymes, and inducible enzymes controlled by catabolite repression. On the basis of this classification, one may conclude that the staphylocoagulase and penicillinase production (Fig. 1, Table 6) by the two bovine strains 20 and 15 is controlled by induction and catabolite repression. The production pattern of staphylocoagulase by strain 104 differed from that of the bovine strains, and is like that of a constitutive enzyme, although the production of the enzyme is relatively low at lower dilution rates and disproportionally high at high dilution rates. The absence of a mode of regulation in strain 104 similar to that in strains 20 and 15, may be due to the fact that the first strain has been subcultured for

more than 30 years *in vitro*, so that regulation has been lost, whereas the bovine strains were isolated only recently. The profile of the production patterns of DNase and phosphatase by strain 15 parallels the total extracellular protein production pattern of strain 20, 15 and 104 and is, as expected, like that of constitutive enzymes. From these data it can be concluded that in strains 20 and 15 the production of staphylocoagulase and DNase, both regarded as the two main criteria for separating *S. aureus* from other staphylococci (Baird-Parker, 1974), are independently regulated.

Catabolite repression of staphylocoagulase production in strain 20 by mannitol could not be demonstrated. This finding is in agreement with those of Arvidson et al. (1976) who found with *S. aureus* strain V8 that the synthesis of phosphatase, protease I and staphylokinase were not under severe catabolite repression. Holme and Arvidson (1976) found increasing levels of phosphatase and staphylokinase with increasing growth rates while protease I was produced with an optimum at a $D = 0.4 \text{ h}^{-1}$. Jarvis et al. (1975) studied the production of extracellular enzymes in continuous cultures of *S. aureus* strain S6 under magnesium- and oxygen-limited growth conditions. They reported that enterotoxin B, lipase, DNase and total extracellular protein increased with increasing dilution rates, whereas lysozyme and β -haemolysin production decreased with increasing growth rates. Moreover, it was found that, with exception of DNase, the production of these enzymes was strongly inhibited by 100 mM glucose.

Besides the growth rate, the following parameters are also important: the concentration of oxygen and of magnesium and the energy state of the cells. It has been reported that a great number of extracellular enzymes is produced optimally by *S. aureus* strains under oxygen-limited growth conditions (Arvidson et al., 1976; Carpenter and Silverman, 1974; Jarvis et al., 1973). From our studies it is clear that staphylocoagulase is produced under similar conditions by all *S. aureus* strains studied. Acetate and carbon dioxide were the predominant end products of the oxidation of mannitol, while high lactate production was only found at very low concentration of oxygen (Fig. 2). Gardner and Lascelles (1962) and Strasters and Winkler (1963) reported acetate and carbon dioxide as main products of glucose metabolism, while 5–10% of the glucose carbon appeared as lactate under aerobic culture conditions. Garrard and Lascelles (1968) demonstrated that lactate dehydrogenase levels and, therefore, lactate production are considerably enhanced under aerobic conditions in the absence of a functional respiratory chain and under aerobic conditions when pyruvate was the sole fermentable carbon source. Thus, in our experiments low oxygen levels could stimulate lactate dehydrogenase activity which resulted in high concentrations of lactate.

Since mannitol was mainly converted to acetate and lactate, and not to cell mass, it is likely that it functioned as an energy source rather than as a carbon source. In the absence of mannitol, the staphylocoagulase production was con-

siderably decreased and this suggests that the energy state of the cell is of great importance in the regulation of staphylocoagulase production. Because increased concentrations of mannitol, acetate or lactate did not inhibit staphylocoagulase production, it was very difficult to indicate which of the other medium constituents could function as a repressor or inducer. Since amino acids could function as energy-, carbon- as well as nitrogen-source, the possibility existed that these components or their metabolites played a role in the regulation of staphylocoagulase production. Although a number of amino acids were depleted during optimal staphylocoagulase production by strain 20, an increase of the casein hydrolysate concentration to 40 mg/ml (so that all amino acids were present in excess of growth requirement) did not lead to an increase of staphylocoagulase production (Table 5). Therefore, it will be difficult to elucidate the exact mechanism of staphylocoagulase production, considering the complex nutrient requirements of *S. aureus* because of which the use of almost complete amino acid mixtures will be inevitable for optimal exoenzyme production (see also Björklind and Arvidson, 1978).

It has been commonly observed that under magnesium limitation, the steady-state bacterial culture density decreased with increasing dilution rates, because the intracellular magnesium requirement varies substantially with the growth rate, being greatest at the maximal growth-rate value (Tempest, 1969). It was, therefore, possible that the decline of the bacterial dry weight curves at increased growth rates was not only due to the limited availability of oxygen alone but also to the magnesium limitation. However, an increase in magnesium concentration did not enhance the bacterial dry weight significantly (Table 4); on the other hand an increase of the stirrer speed from 400 rpm to higher values did not enhance the bacterial dry weight substantially either (Fig. 2). Hence, it seems probable that both nutrients must be available at growth-limiting concentrations.

The nature of the inhibition of staphylocoagulase production by higher concentrations of magnesium as shown in Table 4 is unclear. As far as we are aware there has been only one other report describing the necessity of low magnesium concentrations for the optimal secretion of an enzyme. Bovallius (1969) demonstrated that a magnesium limitation had an adverse effect on biomass production but greatly stimulated the synthesis of a cholinesterase solubilizing factor by a *Cytophaga* sp. Moreover, he showed that cell lysis was not a factor in the release of this enzyme. Although the bulk of the intracellular magnesium is associated with the ribosomes, a small portion is associated with some components of the cell wall and membranes (Meers and Tempest, 1970). Thus, it is possible that magnesium is involved intimately in the permeability of bacterial membranes towards various intra- and extra-cellular substances. A further study is necessary to investigate the precise mode of action of magnesium.

In summary, we conclude that *S. aureus* produces staphylocoagulase under magnesium- and oxygen-limited growth conditions. Optimal production takes

place at submaximal growth rates, limited oxygen availability and at a temperature of 30–31 °C. Two strains of *S. aureus* (15 and 20) produced substantially more staphylocoagulase in chemostat culture as compared with batch cultures of the same organisms. This indicates that chemostat culture, by virtue of its ability to provide constant and specific environmental conditions, may prove to be a better culture technique for the production of this extracellular enzyme. Moreover, it was demonstrated that the production of optimal amounts of staphylocoagulase was not always related to a maximal production of biomass and total extracellular protein.

We wish to thank Dr O. M. Neijssel for helpful discussions and for critically reading the manuscript. We are grateful to Dr J. M. F. Saes for supplying the bovine *S. aureus* strains.

Received 13 August 1980

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SECRETION OF STAPHYLOCOAGULASE BY STAPHYLOCOCCUS AUREUS: THE ROLE OF A CELL-BOUND INTERMEDIATE

SUMMARY

A cell-bound staphylocoagulase could be detected in chemostat cultures of Staphylococcus aureus 104 under magnesium- and oxygen-limited growth conditions. A distribution study revealed that 81% of the enzyme was membrane-bound and could be optimally released by Triton X-100. The remaining part was located in the periplasmic space and was released during protoplasting of the organism. From inhibition studies with cerulenin, quina-crine, lincomycin and chloramphenicol, it was concluded that the cell-bound form was a precursor in the secretion of extracellular staphylocoagulase. The involvement of a lipid intermediate/exoprotein-releasing protease system in the secretion of staphylocoagulase, and of exoproteins in general, is discussed.

INTRODUCTION

The secretion of bacterial exoproteins has been extensively investigated by Lampen and co-workers in the case of penicillinase production by Bacillus licheniformis 749/C. From these studies, a working hypothesis for the export of penicillinase has been proposed (Lampen, 1978). The model postulates the existence of a membrane-bound form of the enzyme which differs structurally from the exopenicillinase in that it has an additional sequence of 24 amino acid residues and a phosphatidylserine residue at the NH₂-terminus (Yamamoto and Lampen, 1976 a, b, c). This strongly hydrophobic tail is considered to anchor the hydrophylic exoenzyme firmly in the membrane. A specific serine protease, located at the outer surface of the cytoplasmic membrane cleaves the covalent bond between the enzyme and the phospholipopeptide moiety, thus allowing the release of the exoenzyme (Aiyappa, Traficante and Lampen, 1977). Consistent with this hypothesis is the finding that

cerulenin, an inhibitor of lipid synthesis (Omura, 1976) and quinacrine, an inhibitor of the penicillinase-releasing protease (Traficante and Lampen, 1977) have a marked effect on penicillinase production by B. licheniformis when added at concentrations which only slightly affect growth (Fishman, Rattem and Citri, 1978; Lampen, 1978). Similar results have been obtained for other extracellular proteins, such as enterotoxin A (Berkeley et al., 1978) and enterotoxins B and C (Altenbern, 1977) of Staphylococcus aureus, levansucrase of Bacillus subtilis (Caulfield et al., 1979) and aminopeptidase of Escherichia coli (Lazdunski, Murgier and Lazdunski, 1979), suggesting a mechanism which is widespread.

Although the production of staphylocoagulase by S. aureus has been subject of many investigations (Altenbern, 1966; Duthie and Haughton, 1958; Engels, Kamps and Van Boven, 1978 and 1980; Fahlberg and Marston, 1960), the cellular precursor of staphylocoagulase is unknown and the biosynthetic pathway leading to its formation has not been established. Altenbern (1966) and Engels et al. (1978) were unable to detect staphylocoagulase in cell lysates, prepared by lyso-staphin treatment of S. aureus. However, fluorescein-labeled antibodies to purified staphylocoagulase attach to the surface of cells of staphylocoagulase-positive staphylococci, demonstrating that this enzyme is present at the cell surface (Blobel and Berman, 1960). Therefore, a study was undertaken to investigate the presumptive presence of a cell-bound staphylocoagulase. In order to elucidate the possible mechanism involved in the secretion of staphylocoagulase, the influence of cerulenin, quinacrine and the protein synthesis inhibitors lincomycin and chloramphenicol on the production of staphylocoagulase in magnesium- and oxygen-limited cultures of S. aureus 104 was studied.

MATERIALS AND METHODS

Organism

Staphylococcus aureus 104 (after Tager) kindly provided by Dr. J.P. Soulier (Centre National de Transfusion Sanguine, Paris) was maintained by monthly subculture on nutrient agar slants.

Growth conditions

Continuous cultures of S. aureus 104 were maintained in casein hydrolysate medium (containing 100 μ M MgCl₂)

in Bioflo C30 chemostats (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) as described by Engels et al. (1980). The dilution rate was $0.28 \pm 0.01 \text{ h}^{-1}$ unless otherwise stated. The pH was maintained at a value of 7.4 by means of an automatic titrator with 2 M KOH as the titrant. The temperature was held at 30°C, the stirrer speed was 400 rpm and the culture volume 320 ml. Silicone antifoaming agent (purchased from BDH) was added automatically as a 2% (w/v) solution at regular time intervals.

Analytical procedures

Bacterial dry weight was measured by the procedure of Herbert, Phipps and Strange (1971). Total extracellular protein was determined, after overnight dialysis of culture supernatants against double glass distilled water at 0-4°C, according to Lowry et al. (1951).

Extracellular staphylocoagulase activity was determined spectrophotometrically with human prothrombin and Chromozym TH (Boehringer Mannheim, W. Germany) as described previously (Engels et al., 1980).

Cell-bound staphylocoagulase activity was determined as follows: 1 ml of culture fluid was centrifuged (5 min at 8000 g) and the cells were washed three times with phosphate buffered saline, pH 7.4 (PBS), containing 50 µg/ml chloramphenicol. After the final washing the cells were taken up in 1 ml of PBS and 100 µl of this cell suspension was added to 500 µl of the assay mixture used for the extracellular staphylocoagulase assay. Subsequently, the staphylocoagulase activity was measured as described above.

Proteolytic activity was determined with azocasein (Sigma) according to Hazen et al. (1965).

Purification of staphylocoagulase

Staphylocoagulase was produced as described by Engels et al. (1978) and purified by chromatography on a bovine prothrombin-Sepharose 4B column according to Igarashi, Morita and Iwanaga (1979).

The purified staphylocoagulase had a specific activity of 2000 U/mg protein.

Preparation of goat anti-staphylocoagulase

Purified staphylocoagulase was precipitated with aluminium hydroxide and resuspended in PBS to a final concentration of 1 mg/ml according to Hudson and Hay (1976). The first intramuscular injection was given with 1 ml precipitated staphylocoagulase mixed with 1 ml

Freund complete adjuvant. A second and third injection (1 mg aluminium hydroxide precipitated staphylocoagulase without Freund adjuvant) was given after intervals of 3 weeks. Seven days after the third injection, blood was collected in 0.1 M sodium oxalate, pH 7.0 and centrifuged at 1200 g for 15 min. To remove procoagulant activity and complement, the supernatant was adsorbed three times with barium sulphate (100 mg/ml) for 30 min at 4°C and heat inactivated at 56°C for 30 min. The adsorbed and inactivated antisera were centrifuged for 30 min at 12000 g at 4°C.

Staphylocoagulase release from washed cells

Cells were harvested from the chemostat, washed three times with PBS containing 50 µg/ml chloramphenicol and resuspended in this buffer plus the releasing agent to be tested. After incubation at 20°C for 30 min with gentle shaking, the cells were centrifuged for 15 min at 15000 g and resuspended in PBS containing 50 µg/ml chloramphenicol. Staphylocoagulase in the supernatants and in the resuspended cells were determined as described above.

Preparation of protoplasts

Cells were harvested from the chemostat, washed three times with PBS containing 50 µg/ml chloramphenicol and suspended in the protoplasting medium containing 4 M NaCl, 50 mM phosphate buffer pH 7.4, 10 mM MgCl₂, 50 µg/ml lysostaphin (Schwarzmann, W. Germany) and 50 µg/ml chloramphenicol. The suspension was incubated with gentle shaking at 20°C for 60 min. The crude protoplasts were subsequently washed with protoplasting medium omitting lysostaphin.

Preparation of membranes

Membranes were prepared according to the following two methods:

- i) Washed protoplasts were suspended in PBS, 10 mM MgCl₂, 50 µg/ml chloramphenicol, 20 µg/ml deoxyribonuclease and incubated at 20°C for 60 min.
- ii) Washed cells were suspended in a mixture containing PBS, 10 mM MgCl₂, 50 µg/ml lysostaphin, 20 µg/ml deoxyribonuclease (Merck, Darmstadt, W. Germany) and 50 µg/ml chloramphenicol and incubated at 20°C for 60 min. The crude membrane fractions were subsequently isolated by centrifugation for 60 min at 48000 g and washing with PBS.

Solubilization of membrane-bound staphylocoagulase

Membranes were suspended in PBS containing 0.1% (w/v) sodium deoxycholate or 1% (v/v) Triton X-100. The suspension was centrifuged for 30 min at 48000 g and the solubilized staphylocoagulase was measured.

Inhibitors

The inhibitors were dissolved in PBS and sterilized by membrane filtration before use. Addition to the chemostat occurred as a single dose to the indicated final concentrations. Quinacrine dihydrochloride and cerulenin were obtained from Sigma Chemical Company (St. Louis, U.S.A.). Chloramphenicol was purchased from Serva (Heidelberg, W. Germany). Lincomycin hydrochloride was a product of Upjohn Company (Kalamazoo, U.S.A.).

RESULTS

Secretion of staphylocoagulase during growth of *S. aureus* 104 in chemostat culture

It has been shown previously (Engels et al., 1980) that in chemostat cultures of *S. aureus* staphylocoagulase was formed optimally under oxygen- and magnesium-limited growth conditions. We have now examined the secretion of this enzyme in these cultures and the possible presence of a cell-bound form. In Table 1 it can be seen that besides an extracellular staphylocoagulase also a cell-bound staphylocoagulase was present. When in the assay of cell-bound staphylocoagulase prothrombin was omitted, hydrolysis of the chromogenic substrate could not be detected indicating that the cell-bound activity was due specifically to staphylocoagulase. Incubation of the cells for 30 min at 37°C with anti-serum to purified extracellular staphylocoagulase completely abolished the cell-bound staphylocoagulase activity whereas preimmune serum did not inhibit at all. It could be calculated that approximately 1.3% of the total extracellular protein was staphylocoagulase and that the amount of cell-bound staphylocoagulase was about 0.3 µg/mg dry weight of cells.

An increase of the dilution rate from 0.28 to 0.55 h⁻¹ resulted in a simultaneous increase of the production rates of extracellular and cell-bound staphylocoagulase, so that the ratio extracellular/cell-bound staphylocoagulase remained constant (data not shown).

Addition of 2% NaCl to the growth medium had a remarkable effect on the amount of cell-bound staphylo-

Table 1. Influence of the sodium chloride and magnesium chloride concentration on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *Staphylococcus aureus* 104 ($D=0.28 \pm 0.01 \text{ h}^{-1}$, 400 rpm, 30°C).

Addition	Bacterial dry weight (mg/ml)	Extracellular staphylocoagulase (U/mg dry wt)	Cell-bound staphylocoagulase (U/mg dry wt)	Ratio extracellular/cell-bound staphylocoagulase	Total extra-cellular protein ($\mu\text{g}/\text{mg}$ dry wt)
None	1.64	1.78	0.56	3.2	69
2% NaCl	1.73	1.68	0.036	47	72
400 μM MgCl_2	1.87	0.29	0.11	2.6	47

coagulase whereas the steady-state concentration of extracellular staphylocoagulase was not affected (Table 1).

Since the production of extracellular staphylocoagulase is inhibited strongly by high concentrations of magnesium (Engels *et al.*, 1980) we investigated the influence of an addition of 400 μ M magnesium chloride to the growth medium on the production of cell-bound staphylocoagulase. It was found that not only the amount of extracellular but also the amount of cell-bound staphylocoagulase was reduced considerably; however, the ratio extracellular/cell-bound staphylocoagulase had decreased only slightly (Table 1).

Release of cell-bound staphylocoagulase from washed cells

The nature of the bond between cell-bound staphylocoagulase and cell was studied by incubation of washed cells of *S. aureus* 104 with various agents under conditions in which the protein synthesis was abolished completely by chloramphenicol (data not shown). Incubation of washed cells with sodium chloride (1, 2, 5, 10, 15, and 24%), potassium chloride (1M), magnesium chloride (1M), sodium citrate (5 and 10%) and EDTA (5 mM) did not show any release of cell-bound staphylocoagulase. Incubation of the washed cells with Tris. HCl, pH 7.4 (1, 2, 5 and 10%) revealed a release of 1, 7, 21 and 37%, respectively. A more effective release of staphylocoagulase could be obtained by incubation of the cells with 0.1% sodium deoxycholate or 1% Triton X-100, which gave a release of 48 and 93% respectively, indicating that the cell-bound staphylocoagulase was bound hydrophobically rather than ionically. The membrane penicillinase of *B. licheniformis* also is not released by high salt concentrations but can be extracted readily by detergents (Sargent and Lampen, 1970).

Localization of cell-bound staphylocoagulase

In order to gain a better insight into the mechanism of the synthesis and secretion of staphylocoagulase it was necessary to determine where the cell-bound form was localized. The cellular distribution of staphylocoagulase in *S. aureus* 104 is shown in Table 2. After preparation of protoplasts, it was observed that 81% of staphylocoagulase activity was associated with the protoplasts, whereas 21% was released and therefore located in the periplasmic space. When the protoplasts were osmotically shocked by resuspension in PBS and

subsequently centrifuged, 69% of the staphylocoagulase activity remained in the particulate fraction and thus apparently membrane-bound. Staphylocoagulase activity in the supernatant i.e. the cytoplasm, could not be detected.

A direct lysis of the cells gave a slight increase of the staphylocoagulase activity. After centrifugation of the lysate, 63% of the activity was found in the membrane fraction, whereas in the supernatant fluids, (containing components of the periplasm plus cytoplasm), 20% of the activity was recovered.

Table 2. Cellular distribution of staphylocoagulase in Staphylococcus aureus 104.

Localization of staphylocoagulase	Staphylocoagulase (units)	Recovery (%)
Cells	6.74	100
Protoplasts	5.48	81
Periplasm	1.40	21
Cell lysate	6.90	102
Cytoplasm	<0.06	< 1
Membranes	4.47	66
1% Triton X-100 membrane extract	3.56	53

Extraction of the membrane fractions with 1% Triton X-100 gave a 53% recovery of the initial activity (80% of the membrane-bound activity). The observed loss of about 20% of the activity during the preparation of membranes, independent of the method used, was probably due to inactivation of the membrane-bound activity, since a direct extraction of whole cells with Triton X-100 gave a release of 93%. The staphylocoagulase activity associated with the protoplasts and the membrane fractions also could be inhibited completely by antiserum to purified extracellular staphylocoagulase.

Effect of cerulenin on the production of cell-bound and extracellular staphylocoagulase

The production of the enterotoxins A, B and C, α -haemolysin and staphylocoagulase by S. aureus is inhibited completely by cerulenin at concentrations that do not affect growth (Altenbern, 1978 and Berkeley et al., 1978). The influence of cerulenin (5 μ g/ml) on the production of cell-bound and extracellular staphylocoagulase in chemostat cultures of S. aureus 104 is shown in Fig. 1. The production of cell-bound staphylocoagulase was completely inhibited whereas the production of biomass was hardly affected. It should be noted that the amount of cell-bound staphylocoagulase decreased at a rate that was twice that of the theoretical wash-out. The latter rate of decay is to be expected when no de novo synthesis and no release of the cell-bound enzyme occurs. The production of extracellular staphylocoagulase was not inhibited instantaneously. After a delay of three hours, a complete inhibition occurred, resulting in a decrease of the concentration of extracellular staphylocoagulase that followed the theoretical wash-out curve. However, the production of total extracellular protein was not affected negatively but even stimulated.

Effect of quinacrine on the production of cell-bound and extracellular staphylocoagulase

Quinacrine has been reported to interfere with the action of the penicillinase-releasing protease of B. licheniformis 749/C (Traficante and Lampen, 1977). The production of levansucrase by B. subtilis (Caulfield et al., 1979) and enterotoxin A by S. aureus (Berkeley et al., 1978) also is inhibited considerably by quinacrine. In chemostat cultures of S. aureus 104, the production of both extracellular and cell-bound staphylocoagulase were inhibited completely by quinacrine at a concentration of 0.1 mM. The production of biomass was slightly affected, whereas no effect on the production of total extracellular protein was observed. The increase in the production of cell-bound and extracellular staphylocoagulase, which occurred three hours after the addition of quinacrine was most probably due to dilution of the inhibitor (Fig. 2).

Although the penicillinase-releasing protease of B. licheniformis 749 has a narrow substrate specificity (Aiyappa and Lampen, 1977), its activity can be demonstrated with casein as the substrate (Aiyappa et al., 1977). However, cell-bound proteolytic activity as well as proteolytic activity in cell lysates of S. aureus 104

Fig. 1 Influence of cerulenin on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* 104 ($D=0.28 \pm 0.01 \text{ h}^{-1}$, 30°C , 400 rpm). Cerulenin was added to the fermenter at time zero to a final concentration of $5 \mu\text{g/ml}$. (●) extracellular staphylocoagulase, (○) cell-bound staphylocoagulase, (■) bacterial dry weight, (□) total extracellular protein (TEP), (---) theoretical washout of non-growing organisms at $D=0.28 \text{ h}^{-1}$.

Fig. 2 Influence of quinacrine on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* 104 ($D=0.28 \pm 0.01 \text{ h}^{-1}$, 30°C , 400 rpm). Quinacrine was added to the fermenter at time zero to a final concentration of 0.1 mM . (●) extracellular staphylocoagulase, (○) cell-bound staphylocoagulase, (■) bacterial dry weight, (□) total extracellular protein (TEP), (---) theoretical wash-out of non-growing organisms at $D=0.28 \text{ h}^{-1}$.

could not be demonstrated with azocasein in the presence of 2 mM CaCl_2 (data not shown).

Fig. 3 Influence of lincomycin on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* 104 ($D=0.28 \pm 0.01 \text{ h}^{-1}$, 30°C , 400 rpm). Lincomycin was added to the fermenter at time zero to a final concentration of $20 \mu\text{g/ml}$. (●) extracellular staphylocoagulase, (○) cell-bound staphylocoagulase, (■) bacterial dry weight, (□) total extracellular protein (TEP), (---) theoretical wash-out of non-growing organisms at $D=0.28 \text{ h}^{-1}$.

Effect of lincomycin and chloramphenicol on the production of cell-bound and extracellular staphylocoagulase

Although the involvement of a lipid intermediate/exo-protein releasing protease system has been accepted generally as a mechanism in the export of exoproteins, it was remarkable that in this study cerulenin and quinacrine did not affect the production of total extracellular protein. Therefore, the "classical" inhibitors of protein synthesis lincomycin and chloramphenicol were used in order to study their effect on the production of total extracellular protein, extracellular and cell-bound staphylocoagulase. Addition of lincomycin to a final concentration of $20 \mu\text{g/ml}$, completely inhibited the production of cell-bound and extracellular staphylocoagulase, whilst the production of total extracellular protein was inhibited for more than 80% (Fig. 3). The

Fig. 4 Influence of chloramphenicol on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* 104 ($D=0.28 \pm 0.02 \text{ h}^{-1}$, 30°C , 400 rpm). Chloramphenicol was added to the fermenter at time zero to final concentrations of $5 \mu\text{g/ml}$ (A), $10 \mu\text{g/ml}$ (B) and $20 \mu\text{g/ml}$ (C), resp. (●) extracellular staphylocoagulase, (○) cell-bound staphylocoagulase, (■) bacterial dry weight, (□) total extracellular protein (TEP), (---) theoretical wash-out of non-growing organisms at $D=0.28 \text{ h}^{-1}$.

Fig. 5 Influence of chloramphenicol on the production of extracellular and cell-bound staphylocoagulase, total extracellular protein and biomass in a chemostat culture of *S. aureus* growing in the presence of 2% NaCl ($D=0.28 \pm 0.01 \text{ h}^{-1}$, 30°C , 400 rpm). Chloramphenicol was added to the fermenter at time zero to final concentrations of $5 \mu\text{g/ml}$ (A) and $20 \mu\text{g/ml}$ (B), resp. (●) extracellular staphylocoagulase, (○) cell-bound staphylocoagulase, (■) bacterial dry weight, (□) total extracellular protein (TEP), (---) theoretical wash-out of non-growing organisms at $D=0.28 \text{ h}^{-1}$.

amount of cell-bound staphylocoagulase decreased at a rate 2.3 times that of the theoretical wash-out, similar to the rate observed with cerulenin treatment (Fig. 1).

The effect of various concentrations of chloramphenicol on the production of cell-bound and extracellular staphylocoagulase, and of total extracellular protein was very remarkable. At a concentration of 5 $\mu\text{g/ml}$ chloramphenicol, a considerable stimulation of the production of extracellular staphylocoagulase took place; the production of cell-bound enzyme and total extracellular protein had increased to a lesser extent (Fig. 4A). In batch cultures of S. aureus 104, however, an almost complete inhibition of the production of extracellular staphylocoagulase was observed at a chloramphenicol concentration of 5 $\mu\text{g/ml}$ (data not shown). When the initial concentration of chloramphenicol was raised to 10 $\mu\text{g/ml}$, a complete inhibition of the production of cell-bound staphylocoagulase was observed (Fig. 4B). The amount of cell-bound staphylocoagulase decreased much more rapidly than the theoretical wash-out rate. The production of extracellular staphylocoagulase was only inhibited during the first two hours of incubation. A subsequent stimulation (comparable to that in Fig. 4A) then occurred after a prolonged incubation time, in which the chloramphenicol concentration had decreased to approximately 5 $\mu\text{g/ml}$, due to wash-out of the antibiotic. Finally, a concentration of 20 $\mu\text{g/ml}$ chloramphenicol enhanced the rate of decrease of cell-bound staphylocoagulase to the same extent as was observed in the case of cerulenin and lincomycin. The inhibition of the production of extracellular staphylocoagulase increased to 100% in the first hour and took place over a prolonged incubation time (Fig. 4C). At concentrations of 10 and 20 $\mu\text{g/ml}$ chloramphenicol, almost no effect on the production of total extracellular protein was observed. Continuous addition of chloramphenicol leading to a constant concentration of the antibiotic in the vessel was not possible because a considerable wash-out of the culture occurred, as can be seen already in the experiments depicted in Fig. 4, after an incubation time of 4-5 h.

Since an addition of 2% NaCl to the growth medium influenced the ratio of extracellular/cell-bound staphylocoagulase (Table 1), it was of interest to study the effect of chloramphenicol under these growth conditions. It can be seen in Fig. 5A that an initial concentration of 5 $\mu\text{g/ml}$ chloramphenicol gave an enormous stimulation of the production of cell-bound and extracellular sta-

phylocoagulase and almost a doubling of the production of total extracellular protein. When the initial chloramphenicol concentration was 20 $\mu\text{g/ml}$, the amount of cell-bound staphylocoagulase decreased very rapidly and disappeared almost completely (Fig. 5B). The production of total extracellular protein decreased considerably (comparable with the inhibition by lincomycin, Fig. 3). The production of extracellular staphylocoagulase decreased almost to the same extent as in the absence of 2% NaCl (Fig. 5B). The degree of inhibition of the biomass production by chloramphenicol was not influenced by the addition of 2% NaCl.

DISCUSSION

The results of the present investigation have resolved the controversy over the existence of an intracellular or cell-bound staphylocoagulase. Besides an extracellular staphylocoagulase, a cell-bound staphylocoagulase was produced in considerable amounts in chemostat cultures of S. aureus 104, under magnesium- and oxygen-limited growth conditions (Table 1). It has to be mentioned that a cell-bound staphylocoagulase could be detected not only in strain 104, but also in various other S. aureus strains. It is probable that the failure in the past to demonstrate cell-bound staphylocoagulase has been caused by the use of cells that did not possess high amounts of the enzyme, along with methods of staphylocoagulase assay that lacked sufficient sensitivity. Although Blobel and Bermann (1960) indeed demonstrated the presence of cell-bound staphylocoagulase by means of immunofluorescence techniques, aspecific binding of the conjugate to protein A (see Forsgren and Sjöquist, 1966) cannot be ruled out in their experiments, since it is not clear from their published data whether the staphylocoagulase-negative S. aureus strain was devoid of protein A. Treatment of washed cells with various agents suggested that the cell associated staphylocoagulase was bound hydrophobically. From the cellular distribution study (Table 2), it can be concluded that the cell-bound staphylocoagulase was predominantly (about 80%) membrane-bound; the remaining part was demonstrated to be present in the periplasmic space whereas cytoplasmic staphylocoagulase could not be detected. Moreover, treatment of the membranes with Triton X-100 resulted in an almost complete release of staphylocoagulase.

Since the cell-bound staphylocoagulase was related antigenically to the extracellular staphylocoagulase and was linked hydrophobically to the cell-membrane, just like the membrane-penicillinase of B. licheniformis, it was reasonable to postulate that the cell-bound form was an intermediate in the secretion process of staphylocoagulase. Therefore, we studied the influence of various inhibitors of the synthesis and secretion processes on the production of cell-bound and extracellular staphylocoagulase.

The pattern of inhibition of cell-bound and extracellular staphylocoagulases, in the presence of cerulenin, was as expected when the synthesis of the cell-bound form was inhibited completely. Thus, the cell-bound staphylocoagulase already formed will be transformed very rapidly into the extracellular form, resulting in a delayed inhibition of production of the extracellular staphylocoagulase (Fig. 1).

However, recent investigations on the structure of cell-bound penicillinase of B. licheniformis (Lampen, 1980 and Simons et al., 1978) revealed that a covalently bound phospholipopeptide is not present but that the membrane-penicillinase contains an amino-terminal peptide extension, hydrophobic in character. Moreover, the production of staphylococcal enterotoxin B by non-growing cells (Altenbern, 1978), and the production of hyaluronidase by S. aureus 722 (Berkeley and Pepper, 1979), cannot be inhibited by cerulenin. Therefore, doubts have been raised about the role of the phospholipid protein intermediates in the export of exoproteins and about the generality of the proposed mechanism. Alternatively, cerulenin might have affected staphylocoagulase levels through non-specific alterations of the membrane backbone, as proposed by Fishman et al. (1978) in the case of levansucrase by B. subtilis, leading to an impeded insertion or transfer of the enzyme molecules through the membrane.

The production of extracellular staphylocoagulase could be inhibited completely by quinacrine suggesting that a proteolytic cleavage step might well be involved in the secretion of staphylocoagulase (Fig. 2). In this connection, it is rather remarkable that the production of cell-bound staphylocoagulase was inhibited completely. However, the rate of decrease of cell-bound staphylocoagulase was considerably lower than that observed in Fig. 1. Therefore, it is likely that a feed-back regulatory mechanism is present.

In general, the use of quinacrine in experiments in-

volving intact cells can be criticised. Although quinacrine is a potent inhibitor of the penicillinase-releasing protease in B. licheniformis, other effects of this compound in biological systems also have been described (reviewed by Sargent, Gosh and Lampen, 1968). Thus, quinacrine is known not only as an inhibitor of many flavine-containing enzymes, adenosine triphosphatases, glucose-6-phosphatase and esterases, but also as an uncoupler of oxidative and photophosphorylation. Since the energy-state of the cell is of great importance in the regulation of production of staphylocoagulase (Engels et al., 1980), the inhibitory effect of quinacrine might be explained on the basis of its ability to uncouple oxidative phosphorylation in S. aureus 104.

No explanation can be given for the absence in S. aureus 104 of a proteolytic enzyme necessary to remove the hydrophobic tail of the cell-bound staphylocoagulase, unless this protease has an unusually narrow substrate specificity. Therefore, further investigations will be necessary to assess the role of such a releasing enzyme in the secretion process of staphylocoagulase and of exoproteins in general.

Although both chloramphenicol and lincomycin affect protein synthesis at the ribosomal level, it can be seen that their effects on the production of cell-bound and extracellular staphylocoagulase, and on total extracellular protein, were rather different (Figs. 3 and 4). Gemmell and Shibl (1976) reported that, in batch cultures of S. aureus, the production of staphylocoagulase and total extracellular protein were inhibited for 100 and 50%, respectively, by lincomycin at concentrations which did not impair growth by more than 20%. In contrast, chloramphenicol inhibited the production of staphylocoagulase only partially while the production of total extracellular protein was not at all inhibited.

The two-fold increase in the rate of decrease of cell-bound staphylocoagulase, compared with that of the decrease of extracellular staphylocoagulase (following the theoretical wash-out rate), in the presence of lincomycin (Fig. 3), supported again the proposal that cell-bound staphylocoagulase is a precursor of extracellular staphylocoagulase in the secretion process. The effects of chloramphenicol on the production of cell-bound and extracellular staphylocoagulase were difficult to interpret. An extra problem in these experiments was that the growth was affected considerably at the chloramphenicol concentrations used.

It can be argued that the membrane-bound ribosomes might be less susceptible to chloramphenicol than the cytoplasmic ribosomes. Thus, low concentrations of chloramphenicol will inhibit the production of cytoplasmic proteins (and partially the growth) and consequently stimulate the production of extracellular proteins. Indeed, this phenomenon can be seen in Fig. 4A. On the other hand, an increase in the chloramphenicol concentration did inhibit the production of cell-bound staphylocoagulase substantially (comparable with the inhibition by lincomycin); however, the production of extracellular staphylocoagulase was inhibited for only 33%, whereas the production of total extracellular protein was not affected (Figs. 4B and 4C).

In Table 1, it can be seen that the addition of 2% NaCl, did not influence the production of extracellular staphylocoagulase in contrast to the amount of cell-bound staphylocoagulase which had decreased almost 16-fold. An increase of the salt concentration generally increases hydrophilic binding to the membrane. Thus, it is probable that the number of hydrophobic anchor places for cell-bound staphylocoagulase had decreased. Therefore, when the cell-bound form is considered as a precursor of extracellular staphylocoagulase, then the turn-over of this precursor has to increase considerably, resulting in a decrease of the steady-state concentration of this protein. Because of this high turn-over of cell-bound staphylocoagulase, under these growth conditions, it is conceivable that addition of chloramphenicol at a stimulatory concentration of 5 $\mu\text{g/ml}$ will lead to a substantial increase of the concentration of cell-bound and extracellular staphylocoagulase (Fig. 5A). On the other hand, when chloramphenicol was added at an inhibitory concentration of 20 $\mu\text{g/ml}$, a dramatic decrease of the cell-bound concentration will be seen, accompanied by a decrease in the production of extracellular staphylocoagulase (Fig. 5B). In the latter case also a decrease in the production of total extracellular protein was observed. Thus, it seemed as if the addition of 2% NaCl to the growth medium, yielded cells in which the turn-over of cell-bound staphylocoagulase, and that of precursors of total extracellular protein, had increased, resulting in an increased susceptibility to chloramphenicol. Nevertheless, more data about the kinetics of the secretion process itself are needed in order to explain the observed phenomena.

Finally, it has been shown that the membrane phosho-

lipid composition in bacilli can be caused to vary extensively by the growth conditions, and that especially under magnesium-limited growth conditions at neutral pH, the content of positively charged lipids is decreased considerably (Minnikin and Abdolrahimzadeh, 1974). Thus, the addition of 400 μ M $MgCl_2$ to the growth medium (Table 1) might have decreased the membrane permeability of S. aureus 104 towards cell-bound staphylocoagulase, resulting in a diminished secretion of the protein.

In summary, we conclude that the hydrophobic cell-bound staphylocoagulase is a precursor in the production of extracellular staphylocoagulase. The effects of cerulenin, quinacrine, lincomycin and chloramphenicol on the production of cell-bound and extracellular staphylocoagulase supported this hypothesis and were in agreement with other observations on other exoproteins. However, with respect to the production of total extracellular protein, it can be concluded that the proposed secretion model for exoproteins, in which a lipid intermediate/exoprotein-releasing protease mechanism is involved, is rather speculative because no influence of cerulenin and of quinacrine could be demonstrated. In order to elucidate the mechanism of secretion of total extracellular protein, and of staphylocoagulase in particular, a complete characterization of the cell-bound precursor(s) and releasing protease(s) is essential before any conclusion can be drawn.

ACKNOWLEDGEMENTS

We wish to thank Dr. J.T.M. Wouters for helpful discussions and Dr. O.M. Neijssel for critically reading the manuscript.

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THE COMPETITIVE ABILITY OF SPONTANEOUS STAPHYLOCOAGULASE-NEGATIVE MUTANTS OF STAPHYLOCOCCUS AUREUS 104 AND NCTC 8178 WITH RESPECT TO GROWTH OF THE PARENT STRAINS IN CONTINUOUS CULTURE

SUMMARY

During the prolonged cultivation of *S. aureus*, strains 104 and NCTC 8178, in continuous culture, staphylocoagulase-negative mutants arose and accumulated progressively in increasing proportions. The resulting loss of production of staphylocoagulase was accompanied by a simultaneous loss of production of α -haemolysin and leucocidin. Characterization of the strains revealed no further differences in biotype, exoenzymes, phage pattern and plasmid content. Cultivation in batch cultures showed that the maximal specific growth rates and specific oxygen consumption rates of the mutant strains were slightly higher than those of the parent strains, whereas the production of total extracellular protein of the mutant strains had decreased significantly. From competition experiments between parent and mutant strains in chemostat cultures at different dilution rates and cultivation temperatures, it was concluded that the underlying mechanism of accumulation of staphylocoagulase-negative mutants in the chemostat is based on differences in affinity for the limiting substrate(s) rather than on differences in the production rates of total extracellular proteins. The complete repression of three exoenzymes, a partial repression of the total extracellular protein production and an increased affinity for the limiting substrate(s) suggested that a mutation in a regulatory gene is involved. The possible role of a transposon in this mutation is discussed.

INTRODUCTION

In the taxonomy of staphylococci, great importance has been attributed to the ability to produce staphylocoagulase (Baird-Parker et al., 1976; Kloos and Schleifer, 1975). However, various authors have reported the spontaneous loss of the staphylocoagulase character

(Duthie and Haughton, 1958; Korman, 1963; Tager, 1974). On the other hand Lotter and Genigeorgis (1977) described spontaneous variants from staphylocoagulase-negative enterotoxigenic staphylococci which had gained the ability to produce staphylocoagulase as well as DNase and α -haemolysin. Tager (1974) suggested that the loss of staphylocoagulase production was due generally to point mutations. Studies, in which the loss or recovery of the staphylocoagulase character was obtained by lysogenic conversion, transduction or mutagenic agents, revealed not only correlations with the loss or recovery of the production of several extracellular enzymes but also with colony form, pigmentation, sensitivity to typing phages, antibiotic resistance, alterations in cell wall composition and the ability to produce acid from a wide variety of sugars (Duval-Iflah et al., 1977; Forsgren et al., 1971; Korman, 1963; Omenn and Friedman, 1970; Yoshikawa et al., 1974). However, the combinations of genetic characters lost or recovered together were different from report to report.

Therefore, it has been postulated, mainly on the basis of studies with pleiotropic exoprotein-negative mutants, that the simultaneous loss or recovery of these characters is due to repression or activation of a common regulatory locus (Björklind and Arvidson, 1980; Forsgren et al., 1971; Omenn and Friedmann, 1970; Yoshikawa et al., 1974).

Models for the regulation of exoprotein biosynthesis at transcriptional level have been formulated by Björklind and Arvidson (1980) and by Coleman, Brown and Stormonth (1975).

It was reported previously (Engels, Kamps and Van Boven, 1980) that during prolonged cultivation of S. aureus, strains 104 and NCTC 8178, under magnesium- and oxygen-limited chemostat growth conditions (optimal for staphylocoagulase production), staphylocoagulase-negative mutants invariably appeared, and out-grew the staphylocoagulase-producing population to more than 90%. Since staphylocoagulase is considered to be an important virulence factor of S. aureus and a major character in the classification of staphylococci, we undertook a detailed investigation into the characteristics of these mutant strains and their competitive ability with respect to their parent strains in chemostat cultures when growing under different conditions. The results, contained in this paper indicate that the pattern of regulation of the production of exoproteins by these staphylocoagulase-negative strains are not compatible with the

proposed models. The probable nature of this mutation and its role in the regulatory mechanism underlying extracellular protein production are discussed.

MATERIALS AND METHODS

Organisms

Staphylococcus aureus, strains 104 and NCTC 8178 were maintained on nutrient agar slopes by monthly subculture. The staphylocoagulase negative (SC⁻) mutants were isolated from chemostat cultures after prolonged cultivation under magnesium- and oxygen-limited growth conditions, as described previously (Engels et al., 1980).

Growth conditions

Batch cultures were grown in 100 ml erlenmeyer flasks containing 20 ml casein hydrolysate, yeast extract or Brain Heart Infusion (BHI) medium in a G-25 rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 190 rpm as previously described (Engels, Kamps and Van Boven, 1978). The incubation temperature was 30°C unless otherwise indicated.

Continuous cultures of S. aureus were maintained in casein hydrolysate medium in Bioflo C30 chemostats (New Brunswick Sci. Co. Inc., New Brunswick, N.J., U.S.A.) as described by Engels et al. (1980). The pH was maintained at a value of 7.4 by means of an automatic titrator with 2 M KOH as the titrant. The temperature was held at 30°C, except otherwise stated. The stirrer speed was 400 rpm and the culture volume was 320 ml. Silicone anti-foaming agent (purchased from BDH) was added automatically at regular time intervals.

Competition experiments in continuous culture

Steady-state cultures of the staphylocoagulase-positive (SC⁺) S. aureus strains were inoculated with S. aureus SC⁻ to a final level of about 5%. Subsequently samples were taken at 3 h intervals during the first 12 hours of growth and at 9-15 h intervals during the following period. After appropriate dilution in 0.15 M NaCl, 0.1 ml-samples were plated on Brain Heart Infusion agar (Difco) plates containing 10% (v/v) citrated human plasma. After 18 h growth at 37°C, the percentage SC⁺ cells in the original samples were estimated from the colony counts. The specific growth rate (μ) of the uncompetitive S. aureus SC⁺ population was calculated using the modified washout growth rate equation from

Jannash (1969) as described by Godwin and Slater (1979): $\mu = [(\ln x_t - \ln x_0) / t_2 - t_1] + D$, where x_0 and x_t are the initial and the final population sizes at times t_1 and t_2 , respectively, and D is the dilution rate maintained during the competition experiment.

Production of revertants

Revertants were produced by treatment of the SC⁻ strains, grown in yeast extract medium, with ethylmethanesulfonate (EMS, Sigma) according to Stobberingh and Winkler (1977).

Analytical procedures

Bacterial dry weight was measured by the procedure of Herbert *et al.* (1971).

Total extracellular protein was determined, after overnight dialysis of culture supernatants against double glass distilled water at 0-4°C, according to Lowry *et al.* (1951).

Maximal specific growth rates ($\mu_{\max}$), specific oxygen consumption rates Q_{O_2} (μ l oxygen consumed/mg dry weight.h) and the production rates of total extracellular protein in batch cultures were measured as follows. Casein hydrolysate medium was inoculated with an 1 in 200 inoculum of overnight cultures and incubated at 30°C as described above. Growth was followed by measuring the culture density spectrophotometrically at 540 nm at 30 min intervals. At the end of the logarithmic phase, the cultures were centrifuged for 20 min at 15000 g. The amount of total extracellular protein was measured in the supernatants; the sediments were washed twice with 50 mM Tris HCl, pH 7.4. The oxygen consumption of the washed cells was measured in a Gilson oxygraph with a Clark electrode in a mixture containing 50 mM Tris. HCl, pH 7.4 and 50 mM mannitol at 30°C. The amount of biomass was determined by dry weight measurement.

Staphylocoagulase (produced in casein hydrolysate medium) was determined as described previously (Engels *et al.*, 1978) by means of a clotting assay with human, rabbit, pig, cow, chicken and dog plasma, respectively. Clotting times up to 4 hours were marked as a positive reaction. Staphylocoagulase positive reactions were only found with human, rabbit and pig plasma.

Haemolysin (produced in BHI medium) was assayed with human, rabbit, and sheep erythrocytes according to Goode and Baldwin (1973). The haemolytic activity of both strains with rabbit, sheep and human erythrocytes was 200, 350 and 50 U/ml, respectively. Therefore, it was

possible that the strains produced a mixture of different haemolysins. However, the haemolytic activity met the criteria of Wiseman (1975) for α -haemolysin (and not for β -, γ - and δ -haemolysin) and was completely abolished by anti α -haemolysin (purchased from Behring Werke AG, Marburg, W. Germany). This led us to the conclusion that the haemolytic activity was due exclusively to α -haemolysin activity and that the remarkable reaction pattern with the different erythrocytes was caused probably by interference of other extracellular products, present in the culture supernatants (Elek and Levy, 1954; Rogolsky, 1979).

Leucocidin (produced in yeast extract medium) was determined with human leucocytes according to Gladstone and Van Heijningen (1957) as described by Van der Vijver (1972).

Protein A was determined with the Aurea A-kit provided by Bio Mérieux (Charbonnières-les-Bains, France).

Protease, Penicillinase, DNase, Lipases (hydrolysis of Tween 20, 40, 60 and 80, respectively), Staphylokinase, Crystal violet reaction and phage typing were carried out as described previously (Engels *et al.*, 1980).

Gelatinase was determined by the method of Clarke (1953).

Hyaluronidase was determined according to Smith and Willett (1968).

Clumping factor was determined by the slide test as described by Cadness-Graves *et al.* (1943).

Catalase activity was determined by adding a few drops of culture in a drop of 3% H₂O₂. Rapid appearance and sustained production of gas bubbles was indicative of a positive reaction.

Plasmids were isolated and characterized according to Wilson, Tatten and Baldwin (1978).

Tellurite reduction and Egg Yolk clearance were determined on Baird-Parker Medium (Oxoid) plates containing 50 ml Egg-Yolk Tellurite emulsion (Oxoid) per litre.

Lysozyme (50 μ g/ml) and lysostaphin (50 μ g/ml) susceptibility were determined as described by Heckzo, Grov and Pulverer (1976).

Penicillin, cephalosporin and novobiocin susceptibility was tested by the routine diffusion plate technique on D.S.T. agar (Oxoid) with the following antibiotics discs (Neo-Sensitabs, provided by A/S Roxo, Toastrup, Denmark): penicillin low (13 μ g per disc), penicillin high (165 μ g per disc), cephalosporin (66 μ g per disc) and novobiocin (100 μ g per disc).

Aerobic production of acid from D-glucose, D-fructose,

D-mannose, maltose, lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, raffinose, D-xylose, saccharose, α -methyl glucoside, N-acetyl glucosamine, production of acetoin, phosphatase, arginine-dehydrolase, urease and the reduction of nitrate were determined by means of the API Staph system (principally based on the scheme of Kloos and Schleifer, 1975) provided by API System S.A. (La Balme-les-Grottes, France).

Aerobic production of acid from D-glucose (also anaerobic), D-mannitol (also anaerobic), lactose, maltose, D-arabinose, D-galactose, D-mannose, D-trehalose, D-xylose, saccharose, production of acetoin, arginine dehydrolase, urease, phosphatase and the reduction of nitrate were also determined by the tube methods according to Baird-Parker (1963).

RESULTS

Accumulation of SC⁻-mutants in the chemostat

Steady-state chemostat cultures of *S. aureus* strains 104 and NCTC 8178 were grown at dilution rates between 0.1 and 0.5 h⁻¹. After a period of 150-250 hours, depending on the growth rate, the amount of staphylocoagulase decreased logarithmically with a half-life of 8-24 hours. Samples taken during this decline were plated on Brain Heart Infusion agar containing human plasma and it could be shown that the decrease of staphylocoagulase production was due to an accumulation of staphylocoagulase-negative mutants. Plating these samples on blood agar plates, indicated that the production of haemolysin was lost simultaneously. It must be mentioned that the moment of appearance of the staphylocoagulase-negative cells was unpredictable and occurred in spite of extensive cloning of the wild type strains on Brain Heart Infusion agar plates containing plasma before inoculation of the chemostat. In batch cultures, SC⁻ cells were observed only after prolonged subcultivation with relatively small inocula (see also Duthie and Haughton, 1958; Tager, 1974).

The accumulation of these mutants suggested that the SC⁻ cells had a significant growth advantage over the SC⁺ cells so that they could outgrow the original SC⁺ population.

In order to obtain more information about the nature and the origin of this growth advantage, an extensive characterization of the parent strains and their mutants was carried out.

Table 1. Biochemical characters of the S. aureus strains and their staphylocoagulase-negative mutants.

	<u>Strain 104</u>		<u>Strain NCTC 8178</u>	
	SC ⁺	SC ⁻	SC ⁺	SC ⁻
Phage group	III	III	I/III	I/III
Plasmids	16MD	16MD	30MD	30MD
Cristal violet reaction	C/D	C/D	C/D	C/D
Protein A	+	+	+	+
Tellurite Egg Yolk reaction	+	+	-	-
Pigment (yellow)	+	+	+	+
Lysozym-susceptibility	-	-	-	-
Lysostaphin-susceptibility	+	+	+	+
Penicillin-susceptibility	+	+	-	-
Cephalosporin-susceptibility	+	+	+	+
Novobiocin-susceptibility	+	+	+	+
Voges-Proskauer	+	+	+	+
Nitrate-reduction	+	+	+	+
Catalase	+	+	+	+
Urease	+	+	+	+
Arginine hydrolase	+	+	+	+
Production of acid from:				
D-glucose (aerobic and anaerobic)	+	+	+	+
D-mannitol (aerobic and anaerobic)	+	+	+	+
D-Mannose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
D-Trehalose	+	+	+	+
D-Melibiose	-	-	-	-
Xylitol	-	-	-	-
Raffinose	-	-	-	-
D-Xylose	-	-	-	-
Saccharose	+	+	+	+
α -Methylglucoside	-	-	-	-
N-Acetyl-glucosamine	+	+	+	+
D-Fructose	+	+	+	+
D-Arabinose	-	-	-	-
D-Galactose	+	+	+	+

Characterization of the *S. aureus* strains

Table 1 shows a large number of biochemical characters of the parent and mutant strains that were examined. The phage groups of parent and mutant strains were identical. Strain 104 and its mutant were sensitive to phage 77 (phage group III), whereas strain NCTC 8178 and its mutant were sensitive to the phages 29, 52 (phage group I) and the phages 53, 54, 75, 77, 84 and 85 (phage group III).

It has been reported that phage conversion can be responsible for the loss of the ability to produce staphylocoagulase (Duval-Iflah et al., 1977; Jollick, 1972; Korman, 1963). Since these conversions were accompanied by a change in sensitivity to typing phages, it is very unlikely that in the *S. aureus* strains, described in this study, the loss of staphylocoagulase production was due to the loss of a prophage.

An analysis of the plasmid contents revealed that the parent strains and its mutant contained identical plasmids in similar amounts.

The remaining characters, like biochemical reactions, susceptibility to lysozyme, lysostaphin and antibiotics, and the fermentation of a variety of sugars did not reveal any difference between parent and mutant strains. An analysis of the exoproteins produced, indicated that, besides staphylocoagulase and α -haemolysin, leucocidin production also was lost (Table 2). Hyperproduction of other exoproteins, as was reported for specific exoprotein-negative strains by Björklind and Arvidson (1980), Coleman (1981) and Yoshikama et al. (1974), was not observed in the SC⁻ strains of *S. aureus* 104 and NCTC 8178.

Determination of the maximal growth rates in batch cultures in 1% casein hydrolysate medium at 30°C (Table 3) showed an increase of $\mu_{\max}$ of about 10% for the mutant strain with a concomitant increase of 6-10% in the specific oxygen consumption rate.

However, the production of total extracellular protein had decreased considerably (34 and 58% for strains 104 and NCTC 8178, respectively).

Production of revertants

Various authors reported that the loss of acquisition of several exoenzymes was due to a mutation in a single locus and therefore revertable by nitrosoguanidine or ethylmethylsulphonate (EMS) (Björklind and Arvidson, 1980; Forsgren et al., 1971; Omenn and Friedman, 1970;

Table 2. Exoenzymes of the S. aureus strains and their staphylocoagulase-negative mutants.

	<u>Strain 104</u>		<u>Strain NCTC 8178</u>	
	SC ⁺	SC ⁻	SC ⁺	SC ⁻
Staphylocoagulase	+	-	+	-
α -Haemolysin	+	-	+	-
Leucocidin	+	-	+	-
DNase	+	+	+	+
Penicillinase	-	-	+	+
Protease	-	-	-	-
Lipases	+	+	-	-
Staphylokinase	+	+	+	+
Clumping factor	+	+	+	+
Phosphatase	+	+	+	+
Gelatinase	+	+	+	+
Hyaluronidase	-	-	-	-

Table 3. Maximum growth rate, specific oxygen consumption rate and total extracellular protein production of the S. aureus strains in batch cultures.

Strain	$\mu_{\max}$ (h ⁻¹)	Q _{O₂} (μ l/h.mg dry wt)	TEP (μ g/mg dry wt)
104 (SC ⁺)	0.77 $\pm$ 0.05) ¹	108 $\pm$ 5	59 $\pm$ 8
104 (SC ⁻)	0.84 $\pm$ 0.05	115 $\pm$ 6	39 $\pm$ 6
NCTC 8178 (SC ⁺)	0.68 $\pm$ 0.06	97 $\pm$ 7	125 $\pm$ 21
NCTC 8178 (SC ⁻)	0.75 $\pm$ 0.07	112 $\pm$ 7	53 $\pm$ 8

)¹ n=5

Fig. 1 Time course of the competition between the parent and mutant strains of *S. aureus* 104 and NCTC 8178, respectively. Cultures of the SC⁺ strains growing in the chemostat at dilution rates of 0.17 h⁻¹ and 0.50 h⁻¹ were inoculated with SC⁻ organisms to a level of 1% SC⁻. (●) actual loss of SC⁺ organisms at D=0.17 h⁻¹, (---) theoretical wash-out of non-growing organisms at D=0.17 h⁻¹, (■) actual loss of SC⁺ organisms at D=0.50 h⁻¹, (---) theoretical wash-out of non-growing organisms at D=0.50 h⁻¹.

Table 4. Competition experiments between staphylocoagulase-positive and staphylocoagulase-negative *S. aureus* strains at different dilution rates in chemostat cultures (400 rpm, 30°C).

Strain	Dilution rate (h ⁻¹)	Specific growth rate μ of the SC positive population	Percentage decrease of μ for SC positive population
104	0.17	0.13	21.9
	0.34	0.29	14.4
	0.50	0.47	5.4
	0.60	0.60	0.8
	0.70	0.70	0.0
NCTC 8178	0.17	0.13	21.2
	0.35	0.29	17.7
	0.50	0.43	13.2
	0.60	0.55	8.2
	0.70	0.70	0.0

Yoshikawa *et al.*, 1974). Several attempts were made with EMS as mutagenic agent to obtain SC⁺ revertants from both of the SC⁻ mutant strains. Only in a few cases were SC⁺ revertants found, but after subculturing on BHI agar medium containing human plasma, SC⁺ colonies could not be demonstrated indicating that these revertants did not possess a stable staphylocoagulase character. Reversion of the production of haemolysin and leucocidin was never observed. The lack of a selective medium needed to enrich the SC⁺ cells was one of the major problems in these reversion experiments. In practice, it was not possible to screen more than about 30,000 colonies. Therefore, it is possible that revertants easily could have been missed when the frequency of reversion was low. Alternatively, the possibility that a single mutation is not involved at all, cannot be excluded.

Competition experiments between parent and mutant strains

Since the moment of appearance of the staphylocoagulase-negative cells in chemostat culture was unpredictable, the following approach was chosen. Continuous cultures of the SC⁺ strains were set up and were deliberately contaminated with SC⁻ cells as soon as the SC⁺ population had reached a steady-state.

In Fig. 1 the time course of these competition experiments at dilution rates of 0.17 and 0.50 h⁻¹ are shown. Immediately after the addition of the SC⁻ cells to SC⁺ steady state populations, the competitive advantage of the former was evident. The differences in the growth dynamics of the competing populations were the same as in chemostat cultures in which a spontaneous loss of staphylocoagulase production had occurred (half life of 8-24 hours). The competitiveness between the two populations expressed as percentage decrease in μ for the SC⁺ cells in relation to the dilution rate is shown in Table 4. At a dilution rate of 0.17 h⁻¹ a considerable competition was observed. The competitiveness decreased with increasing dilution rates, whereas at a D=0.70 h⁻¹ (almost $\mu_{\max}$) no competition was evident. The influence of the cultivation temperature on the competitiveness is shown in Table 5. The SC⁺ populations were grown at a dilution rate of 0.35 h⁻¹. The decrease in numbers of SC⁺ cells was followed at cultivation temperatures of 25, 30, 35 and 40°C, respectively. It can be seen that the competitiveness increased with increasing temperatures. Generally, the decrease in μ for the SC⁺ cells in these competition experiments was of the same magnitude

Table 5. Competition experiments between staphylocoagulase- positive and staphylocoagulase-negative *S. aureus* strains at different temperatures in chemostat cultures ($D=0.35\pm0.01\text{ h}^{-1}$, 400 rpm).

Strain	Temperature (°C)	Percentage decrease of μ for SC positive population
104	25	0.5
	30	14.4
	35	18.0
	40	26.3
NCTC 8178	25	10.2
	30	17.7
	35	23.2
	40	28.0

Fig. 2 The relation between the percentages decrease of μ for the SC⁺ population of *S. aureus* strain 104 and NCTC 8178, respectively and their relative growth rates. The percentages decrease of μ for the SC⁺ cells at different dilution rates (O) and at different temperatures (●) were obtained from Tables 4 and 5, respectively.

of the increase in μ for the SC⁻ cells (data not shown). In some experiments the SC⁺ population did not disappear completely but persisted in the culture at a low percentage (about 1%). This phenomenon may be due to wall growth or non-homogeneous conditions in the culture vessel (see also Godwin and Slater, 1979; Melling, Ellwood and Robinson, 1977; Wouters *et al.*, 1980). Tempest (1976) pointed out that growth rate per se is not a physiologically relevant parameter but merely one manifestation of the steady-state concentration of the growth limiting nutrient. An increase in the cultivation temperature generally leads to an increase in $\mu_{\max}$, so that the concentration of the limiting nutrient(s) will decrease. Therefore, one can only compare two steady-state cultures growing at the same dilution rate when the steady-state concentrations of growth-limiting nutrient are approximately equal. Because of analytical difficulties, it is practically rather complex to obtain reliable data on the steady-state extracellular concentrations of growth-limiting nutrient at values close to the value of K_s (the affinity constant for the limiting substrate). Therefore the concept of relative growth was employed as described by Evans (1976) and Tempest (1976). For this purpose the assumption is made that at the temperatures studied, K_s remained constant. Subsequently the dilution rates were corrected for the maximal growth rates which were readily obtained from batch growth curves in casein hydrolysate medium at the different temperatures used (data not shown).

In Fig. 2, the competitiveness of both strains obtained from Tables 4 and 5, were plotted against their "relative" growth rates; that is, the ratio of the imposed growth rate to the maximum growth rate ($\mu/\mu_{\max}$). It can be seen that there is a rather close correlation in the range of relative growth rates between 0.30-0.70 in the case of strain 104 and of 0.40-1.0 for strain NCTC 8178. Thus, it seems reasonable to conclude that the influence of the temperature on the competitiveness indeed reflected an increase in $\mu_{\max}$, resulting in a decrease in the growth limiting nutrient concentration required to support growth at some sub-maximal value.

Influence of dilution rate on the total extracellular production of the parent and mutant strains

The specific rates of total extracellular protein production, as depicted in Fig. 3, increased linearly with increasing dilution rates. It can be calculated that the ratio of the production rates of the SC⁺ and

the SC⁻ cells of both strains was rather constant in the range of the dilution rates studied. Since the competitiveness between the parent and mutant strains decreased with increasing dilution rates (Table 4), it is unlikely that the diminished total extracellular protein production played an important role in enhancing the growth advantage of SC⁻ strains over the SC⁺ strains.

Fig. 3 Influence of the dilution rate on the specific rates of production of total extracellular protein (TEP) in chemostat cultures of the parent and mutant strains of *S. aureus* 104 and NCTC 8178, respectively. (▲) *S. aureus* strain 104 (SC⁺), (△) *S. aureus* strain 104 (SC⁻), (●) *S. aureus* strain NCTC 8178 (SC⁺), (○) *S. aureus* strain NCTC 8178 (SC⁻).

DISCUSSION

Cultivation of *S. aureus* strains 104 and NCTC 8178 in continuous culture gave rise to staphylocoagulase-negative (SC⁻) cells which subsequently accumulated by virtue of their apparent growth advantage under the imposed growth conditions. Characterization of these mutants revealed that they differed from their parent strains in only a few characters. Apart from a complete loss of ability to produce staphylocoagulase, α -haemolysin and leucocidin (Table 2), the production of total extracellular protein had decreased considerably (Table 3, Fig. 3). In competition experiments, it was shown that the competitiveness between mutant and parent

strain increased with decreasing dilution rates and with increasing cultivation temperatures (Tables 4 and 5). These data therefore raise two important questions: firstly, which factors are involved in promoting the observed growth advantage of the SC⁻ over the SC⁺ cells? And secondly, in what way does this mutation affect the regulatory mechanism of extracellular protein synthesis.

With regard to the first question, there are many factors that affect this growth advantage. One of these might be the excessive burden carried by the SC⁺ cells in synthesizing considerable amounts of extracellular proteins. However, the competitiveness decreased with increasing dilution rates (Table 4), in contrast to the production rate of total extracellular protein which increased with increasing dilution rates to the same extent for the mutant as well as the parent strains (Fig. 3). Thus, it is unlikely that this factor is of over-riding importance in the competition. Another factor might be the difference in maximum specific growth rate, as was observed by Godwin and Slater (1978) when studying the competition between plasmid-containing and plasmid-free cells. The slight increase in maximal growth rate and maximal oxygen consumption rate (Table 3) are not sufficient to account for the observed selection of SC⁻ cells during the competition experiments in continuous culture. More decisive for the outcome of a competition experiment at submaximal specific growth rates, is the difference in affinity for the limiting substrate(s) of the competitors (Harder, Kuenen and Matin, 1977). The competition studies with the parent and mutant strains of *S. aureus* 104 and NCTC 8178 showed that the growth advantage of the mutant strains increased with a decrease in the concentration of the growth limiting substrates, i.e. at low growth rates or, when the growth rate was kept constant, at high growth temperatures (Tables 4 and 5). This is illustrated clearly once again in Fig. 2, in which the competitiveness is plotted against the relative growth rate. Therefore, it is reasonable to conclude that an increased affinity for the limiting substrate is the decisive factor in the competition between the SC⁻ and SC⁺ cells.

In view of the strongly selective growth conditions occurring in chemostat cultures, it is clear why SC⁻ cells were not observed previously in batch cultures (Engels *et al.*, 1978). During the exponential growth phase both populations coexisted and only at the end of this phase, the nutritional restriction became manifest, resulting in an increased competition between parent and

mutant strain. Since the number of generations was low, due to relatively large inocula used, SC⁻ cells were not observed. Therefore, SC⁻ cells will only accumulate in batch cultures after prolonged subculturing, using very small inocula and high growth temperatures (Duthie and Haughton, 1958; Tager, 1974).

Coleman et al. (1975) postulated a regulatory model for exoenzyme biosynthesis in which the size of the pool of nucleic acid precursors controls exoprotein-associated RNA synthesis and thus exoenzyme formation. This accumulation of precursors is thought to be restricted to conditions of nutritional limitation, occurring in batch cultures after termination of exponential growth, and in chemostat cultures at submaximal dilution rates. Although this phenomenon was observed in batch cultures of S. aureus (Abbas-Ali and Coleman, 1977; Coleman and Abbas-Ali, 1977), it was demonstrated with chemostat cultures of Bacillus licheniformis 749/C, and with several strains of S. aureus, that the production rate of total extracellular protein increased with increasing dilution (= growth) rate (Engels et al., 1980; Jarvis, Laurence and Pritchard, 1975; Wouters and Buysman, 1977 b). Furthermore, the observation of Coleman (1981) that the loss, or decrease, of the production of some exoproteins by pleiotropic mutants of S. aureus was compensated by an increase in the production of other exoproteins, so that the total production rate of exoprotein was not affected, could not be confirmed in this study. Therefore, an additional regulatory mechanism is necessary in order to understand why certain exoenzymes are only produced under certain conditions of nutritional limitation (see also Wouters and Buysman, 1977 a). A model that accounts for exoprotein synthesis during exponential growth, as well as during post-exponential growth of S. aureus, was proposed by Björklind and Arvidson (1980). Factors like catabolite repression and end-product repression were also involved by introducing a signal substance S (i.e. an intermediate metabolite) which activates a hypothetical protein EX. This protein which is supposed to be produced throughout the growth cycle, is needed in addition to the RNA polymerase for the transcription of structural and repressor genes. The different patterns of exoprotein production in the spontaneous mutants of S. aureus, strain V8 were explained in terms of different affinity between EX-S complex or EX-S-RNA polymerase and the individual promotor regions. These spontaneous mutations (probably in factor EX or in some enzyme involved in the synthesis of S)

could be reverted to the wild type in one step by treatment with NG. It was concluded therefore that the multiple changes of the mutants were due to a single mutation and that a superior/regulatory gene was involved. The decrease in the production of proteinases I and III, α -haemolysin, staphylokinase, DNase and phosphatase and the simultaneous increase of the production of staphylocoagulase and protein A could be explained with this model. The spontaneous mutations in S. aureus strains 104 and NCTC 8178 yielded a simultaneous loss of staphylocoagulase, α -haemolysin and leucocidin. Moreover, the total extracellular production rate had decreased considerably. Although a mutation in the regulatory gene is likely to be involved in these strains, it was impossible to obtain a reversion of these mutants to the wild strains with EMS.

Brown and Pattee (1980) reported that the spontaneous loss of α -haemolysin production in various S. aureus strains also was not reversible with mutagenic agents, and that the loss of a prophage or a plasmid was not involved. From a detailed genetic study it was concluded that the regulation of α -haemolysin production was located on a transposable genetic element. These elements can encode sequences that behave as transcriptional start and stop signals and can act as supernumerary regulation switches affecting gene repression. Furthermore, depending upon the orientation of the transposable element, the function of the mutated gene also may be abolished or, in addition, the expression of promotor distal genes in an operon can be either eliminated or enhanced. Therefore, transposable elements might be largely responsible for the spontaneous chromosomal alterations in many bacteria (Kopecko, 1980). Although a detailed genetic analysis is necessary, the involvement of such an element in S. aureus strains 104 and NCTC 8178 clearly would explain the size of the mutation (complete loss of three exoenzymes, decreased production rate of total extracellular protein and an increased affinity for the limiting substrates) and the fact that the observed mutation was not reversible with a mutagenic agent.

Generally, the presence of transposable genetic elements which may or may be not site specific, would provide in combination with phage conversion (Duval-Iflah et al., 1977; De Waard and Winkler, 1963) a framework to elucidate the widely described phenomenon of pleiotropy in S. aureus.

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RAPID AND DIRECT STAPHYLOCOAGULASE ASSAY THAT USES A CHROMOGENIC SUBSTRATE FOR IDENTIFICATION OF STAPHYLOCOCCUS AUREUS

SUMMARY

A rapid and direct assay of staphylocoagulase that uses chromogenic substrate Chromozym TH was developed for the routine identification of Staphylococcus aureus. False-positive and false-negative reactions were eliminated by measuring the activity of the staphylocoagulase-prothrombin complex directly. The method was compared with three clotting assays. The results show that the new assay is specific, quantitative, and easily automated. It offers clinical laboratories a more reliable assay of staphylocoagulase and is a valuable aid for the rapid identification of S. aureus.

INTRODUCTION

The ability to produce staphylocoagulase is the most widely used and generally accepted criterion for identification of Staphylococcus aureus (3, 12). Most clinical laboratories perform a staphylocoagulase assay that is based on the ability of the enzyme to clot plasma. The clotting action of staphylocoagulase is due to a specific and stoichiometric reaction of staphylocoagulase with prothrombin, yielding a complex, i.e., staphylo-thrombin, which converts fibrinogen into insoluble fibrin in a manner similar to the action of physiologically formed thrombin (10, 23). Most assay methods routinely used are qualitative methods. Apart from difficulty in standardizing and quantitating these assays, false-negative and false-positive reactions may occur. False-negative reactions will take place if fibrinogen and fibrin are partially cleaved by proteases, staphylokinase-induced plasmin, or both, so that an insoluble fibrin clot cannot be formed or is dissolved (21). On the other hand, false-positive reactions are observed if prothrombin is activated by trypsin or papain-like proteases, which are present in the culture fluid of S. aureus (20). Recently, prothrombin activa-

tion by metalloproteases has also been described (20). The present study describes a direct assay of the activity of staphylothrombin based on limited proteolysis of a synthetic chromogenic substrate. The method is quantitative and can be automated easily. A comparison of this method with "classic" clotting assays was made by using recently isolated clinical strains of S. aureus.

MATERIALS AND METHODS

Bacterial strains. S. aureus strains (n=70) recently isolated from various clinical sources were kindly provided by W.J. van Leeuwen, Department of Bacteriology, National Institute for Public Health, Bilthoven, The Netherlands. The strains were characterized as S. aureus on the basis of phage and biotyping according to the method of Blair and Williams (4) and Kloos and Schleifer (12), respectively. For biochemical characterization, the API Staph system (API System S.A., La Balme les Grottes, France) was used. Another 20 clinical isolates with the morphological characteristics of Staphylococcus spp. (yellow-white colonies with a smooth glistening surface and staining as gram-positive grapelike clusters) were also tested. These strains were nontypable with the International Phage Typing set and were not clearly identified to the species level according to the (restrictive) manufacturer's manual of the API Staph system. For controls, the following staphylocoagulase-negative reference strains were used: Staphylococcus haemolyticus SM102, Staphylococcus intermedius CF238, Staphylococcus hyicus CBCC1462 (obtained from W.E. Kloos, Department of Genetics, North Carolina State University, Raleigh), and Staphylococcus epidermidis 407 (obtained from J. Verhoef, Department of Medical Microbiology, University of Utrecht, The Netherlands).

Prothrombin-proconvertin-Stuart-Prower factor-anti-hemophilic factor B (PPSB). A concentrate of the blood clotting factors II, VII, X, and IX was prepared according to the method of Soulier et al. (16).

Cultivation conditions. The organisms were cultivated statically in test tubes (16 by 100 mm) containing 5 ml of nutrient broth (Difco Laboratories, Detroit, Mich.) or brain heart infusion medium (Difco) at 37°C for 18 h. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.2% (wt/vol) to these media to study its effect on staphylocoagulase production.

Deoxyribonuclease. Deoxyribonuclease production was screened on agar plates containing 1% deoxyribonucleic acid (Difco), as described in the manufacturer's manual.

Protease. Protease production was determined with azocasein (Sigma) according to the method of Hazen et al. (9).

Staphylocoagulase. Staphylocoagulase was produced from S. aureus strain 104, as described by Engels, Kamps, and Van Boven (6), and purified by chromatography on a bovine prothrombin-Sepharose 4B affinity column according to the method of Igarashi, Morita, and Iwanaga (11).

Staphylocoagulase assays.

(i) Chromogenic substrate method. After centrifugation of the cultures at $2,500 \times g$ for 15 min, 30 μ l of supernatant fluid was mixed with 120 μ l of reagent mixture containing 72 mM triethanolamine buffer (pH 8.4), 144 mM NaCl, 166 μ M Chromozym TH (Boehringer, Mannheim, West Germany) and 5 μ l of human PPSB solution containing 50 μ g of prothrombin per ml as the prothrombin source. In this assay, staphylocoagulase activates prothrombin to staphylothrbin. The thrombin-like activity of this complex cleaves the synthetic tripeptide Chromozym TH (N-Tosyl-glycyl-L-prolyl-L-arginyl-p-nitroanilide.hydrochloride) by limited proteolysis, liberating the yellow p-nitroaniline (18,22). The reaction was performed in microtiter plates (Greiner, Nuertingen, West Germany). After incubation for 1 and 2 h at 37°C, the absorbances were measured with a Titertek Multiskan (Flow Laboratories Ltd., Irvine, United Kingdom) at 405 nm and were corrected automatically for blank medium reactions. Since the staphylocoagulase-negative reference strains gave absorbances lower than 0.05 after 1 h of incubation, absorbances in the range of 0 to 0.05 were recorded as negative reactions, and absorbances exceeding the value of 0.05 were recorded as positive. One unit of staphylocoagulase was defined as the amount of enzyme giving an increase in absorbance at 405 nm of 1.0 per min at 37°C.

(ii) Tube inoculation method. Twenty-five microliters of the culture fluid was inoculated into 0.5 ml of citrated human plasma and incubated at 37°C. After 1 and 4 h, the mixtures were screened for clotting. Any degree of clotting constituted a positive reaction. Flocculent or fibrous precipitates were recorded as negative (13).

(iii) Tube supernatant fluid method. After centrifugation of the cultures at $2,500 \times g$ for 15 min, 0.1 ml of supernatant was mixed with 0.1 ml of citrated human

Fig. 1 Time course of the hydrolysis of Chromozym TH with different amounts of staphylocoagulase. The reactions were performed in microtiter plates at 37°C with different amounts of purified staphylocoagulase, as described in the text. The final concentrations in the reaction mixtures were: (●) 81 ng/ml, (○) 68 ng/ml, (▲) 54 ng/ml, (△) 41 ng/ml, (■) 27 ng/ml, (□) 14 ng/ml.

Fig. 2 Standard curve of the linear relationship between the different staphylocoagulase concentrations and their corresponding reaction rates, calculated from the time courses shown in Fig. 1.

plasma and incubated at 37°C. After 1 and 4 h, the mixtures were screened for clotting according to the criteria described for the tube inoculation method.

(iv) Staphylocoagulase plates. In a method comparable to those described by Van der Vijver, Kraayeveld, and Michel (19) and Parisi, Baldwin, and Sottile (15), brain heart infusion agar plates containing 10% (vol/vol) citrated human plasma were used. After inoculation and incubation at 37°C, staphylocogulase production was indicated by opalescent haloes (diameter, 4 to 10 mm) surrounding the colonies.

RESULTS

It has been shown previously (7) that a direct determination of staphylothrombin activity can be performed by using a chromogenic substrate. In this assay method a linear relationship was obtained not only between the incubation time and the hydrolysis of Chromozym TH with different amounts of purified staphylocoagulase (Fig. 1) but also between the concentrations of staphylocoagulase and their corresponding reaction rates (Fig. 2). Linearity of the activities was observed up to absorbances of 0.9 to 1.0. In this experiment the amounts of purified staphylocoagulase were far in excess of what was found in the supernatant fluids of the staphylococcal cultures, which gave absorbances in the range of 0 to 0.5.

The influence of the cultivation medium on the production of staphylocoagulase by the S. aureus strains (n=70), when assayed by the chromogenic substrate and the tube clotting methods, is shown in Table 1. The percentages of staphylocoagulase-positive reactions of the tested strains, subcultured in brain heart infusion medium, were 84.3, 90.0 and 84.3% with the chromogenic substrate method, the tube incubation, and the tube supernatant fluid method as the respective assay methods. After a prolonged incubation time, the percentages increased to 97.1, 98.6, and 100%, respectively. The staphylocoagulase production of one strain was variable and therefore resulted in a 98.6% - instead of a 100% - positive score for the tested organisms. The amount of staphylocoagulase produced by the strains after subculturing in nutrient broth was much lower, resulting in only 10.0, 40.0 and 35.7% positive reactions, respectively, when the assay methods mentioned above were used. A longer incubation time resulted in an increase to 40.0, 82.9, and 88.6%, respectively.

For achieving high sensitivity of the assays, the amount of staphylocoagulase produced in the 18 h cultures had to be optimized. We achieved optimization by adding bovine serum albumin to a final concentration of 0.2% (wt/vol), as recommended by Altenbern (2).

Table 1. Comparison of different assays of staphylocoagulase produced by seventy strains of S. aureus in relation to various cultivation media.

Cultivation medium	% Positive staphylocoagulase reactions at indicated assay incubation time					
	Chromogenic substrate method		Tube inocula- tion method		Tube supernatant method	
	1 h	2 h	1 h	4 h	1 h	4 h
Brain heart infusion	84.3	97.1	90.0	98.6	84.3	100
Brain heart infusion + 0.2% bovine serum albumin	98.6	98.6	97.1	100	98.6	98.6
Nutrient broth	10.0	40.0	40.0	82.9	35.7	88.6
Nutrient broth + 0.2% bovine serum albumin	74.3	97.1	54.3	87.1	52.9	97.1

Addition of bovine serum albumin did increase the amount of staphylocoagulase produced. The effect was most obvious after subculturing in nutrient broth using the chromogenic substrate method. An increase in positive reactions from 40 to 97.1% was observed.

Staphylocoagulase production on brain heart infusion agar plates containing plasma showed that only 51 of the 70 S. aureus strains (72.9%) gave positive reactions. In 11 strains, very weak or no staphylocoagulase reactions were observed. Moreover, interference by fibrinolysis took place with eight strains; thus, these strains also had to be scored as staphylocoagulase negative. Therefore, misjudging of the staphylocoagulase reaction in the latter strains cannot be excluded. Of the staphylocoagulase-negative control strains, S. epidermidis and S. haemolyticus were determined to be staphylocoagulase negative by all the test methods. S. hyicus and S. intermedius gave a positive reaction in the clotting

assays, whereas when the strains were assayed by the chromogenic substrate method, positive reactions were not observed. It was demonstrated that S. hyicus produced a proteolytic enzyme, which may explain the false-positive reaction in the clotting assays.

The additional 20 staphylococcal strains produced only trace amounts of staphylocoagulase in the clotting assays. However, in the chromogenic substrate assay, four strains gave a positive reaction which was not inhibited by 5 mM ethylenediaminetetraacetic acid (EDTA). The additional positive deoxyribonuclease reaction indicated that these four strains could be identified as S. aureus.

Because false-positive reactions could have been caused by the presence of metalloproteases in the S. aureus strains, the chromogenic substrate assay was performed also in the presence of 5 mM EDTA, as described by Wegrzynowicz (20). For this assay, no influence of EDTA addition on the staphylocoagulase reaction could be demonstrated.

For routine use in a clinical laboratory, it is preferable to complete the identification within 1 day. Therefore, the cultivation time of the strains in brain heart infusion medium supplemented with 0.2% bovine serum albumin was reduced from 18 to 4 h. In spite of lower staphylocoagulase activities (data not shown), 98.6% of the chromogenic substrate assay tests still gave positive reactions.

DISCUSSION

Staphylocoagulase production has been established as one of the definitive characteristics of S. aureus (3). However, rapid identification of S. aureus has not always been possible because most assays may take 24 h to produce a positive result. In general, two tests can be performed: a tube test and a slide test. Although slide tests are performed in various laboratories, it must be emphasized that this assay involves the clumping factor and not staphylocoagulase activity (1).

The classic clotting methods were very reliable, as expected (Table 1). However, when the criteria of Sperber and Tatini (17) were strictly followed, a considerably lower number of positive reactions were recorded in the clotting assays because, for about 15% of the strains, no firm opaque clots were formed after 4 h, owing to fibrinolysis and related reactions. For this

reason, longer assay times should be avoided also (13). Therefore, misjudging of the reaction in the clotting assays can occur easily if one has no experience in this field.

The staphylocoagulase plate assay, which is in principle comparable to the method described by Van der Vijver et al. (19), and the quantitative radial slide assay method described by Kohl and Johnson (14) did not yield very reliable results. The relatively low numbers of positive staphylocoagulase reactions observed (72.9%), were probably due to fibrinolysin or related enzymes produced by the S. aureus strains. Kohl and Johnson might not have observed this interfering enzyme reaction because, in contrast to the strains used in this study, their test strains were from food rather than clinical sources.

Although false-negative and false-positive reactions in the clotting assays can be prevented by using a mixture of different protease inhibitors (20, 21), an assay based on a direct and specific measurement of staphylo-thrombin activity is preferred. From this study, it can be concluded that the chromogenic substrate assay is more reliable than the clotting assays. Even with the 20 weak staphylocoagulase-producing staphylococcal strains, staphylocoagulase could be demonstrated positively in four strains. The assay is specific, since S. intermedius and S. hyicus will not give a positive result. Staphylocoagulase production by S. intermedius and S. hyicus, as reported by Hájek (8) and Devriese (5), needs further investigation.

Cultivation of the S. aureus strains in brain heart infusion medium supplemented with 0.2% bovine serum albumin and an assay time of 1 h resulted in a 98.6- to 100%-positive score (Table 1). For routine assays, a cultivation time of 4 h enables a staphylocoagulase assay within 1 day. To eliminate possible false-positive reactions, we recommend adding 5 mM EDTA to the assay mixture. PPSB can be obtained from almost every blood transfusion center at reasonable prices, especially in the case of outdated samples. Analyzing the data quantitatively, we could not observe a correlation between the amount of staphylocoagulase produced and phage type or origin of the S. aureus strains. Also, there was no correlation between origin and phage type.

Nevertheless, this test enables a quantitative assay of staphylocoagulase and provides the means for setting up an epidemiological survey with large numbers of S. aureus strains to investigate a possible relationship

between the amount of staphylocoagulase produced and the virulence of S. aureus on the one hand and the role of the enzyme in the pathogenesis of S. aureus on the other. In addition, the availability of a simple quantitative method for the assay of staphylocoagulase makes it possible to monitor staphylocoagulase activities during enzyme purification. Since the assay can be performed in microtiter plates, high numbers of samples can be tested automatically.

In conclusion, the major advantages of this test are not only the short assay time but also the possibility of screening large numbers of strains or staphylocoagulase samples in a quantitative and specific way.

ACKNOWLEDGEMENTS

We are grateful to W.J. van Leeuwen for providing the Staphylococcus strains used. We thank E.E. Stobberingh for helpful discussions and for critically reading the manuscript.

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GENERAL DISCUSSION AND SUMMARY

Staphylococcus aureus is a commensal micro-organism of low virulence which usually causes severe infections only when the normal defences of the host are impaired. The acute inflammatory response and the activity of phagocytic cells are the most important defence mechanisms against staphylococcal infection. On the other hand, S. aureus has the disposal of a variety of virulence factors such as cell-wall components and extracellular proteins which can interfere strongly with the defence mechanisms of the host. Although our present knowledge of the interaction between antigenetic structure, opsonins and phagocytic cells has been enriched considerably by the work of Verbrugh and co-workers, the role of extracellular proteins in the pathogenesis of S. aureus is still not well understood. It has to be noted that generally, studies concerning the virulence of S. aureus have been greatly hampered by the lack of availability of an appropriate animal model. Not only do relatively high doses of micro-organisms have to be applied but also the type of S. aureus strain, the route of administration and the animal species play an important role in the persistence of S. aureus during the infection process. Experimental staphylococcal infections of the human skin pose great difficulties since a balance has to be found between a failure to produce any infection, and a spreading and severe infection, which is therefore unacceptable.

For these reasons, it was proposed by Musher to choose animals with an underlying disease or an impaired host defence mechanism as subjects for a study of the pathogenesis of S. aureus.

Although this approach might be successful, the number of variables in the infection process will be still considerable. An alternative model can be found probably in the assay system, described by Verhoef *et al.* This assay employs tritiated thymidine labeled staphylococci and permits a quantitative study of the separate steps of bacterial opsonization, attachment, ingestion and intracellular killing. In a similar model the physiological importance of exoproteins of S. aureus could be investigated.

Since there is substantial evidence indicating that

changes in the growth conditions of S. aureus induce major alterations in antigenic structure, production of exoproteins and biochemical properties of S. aureus, due to phenotypical variations, it is progressively becoming recognized that the virulence of S. aureus varies according to the environment in which the staphylococci grow.

In the last decades much attention has been paid to the production and the characterization of the exoproteins of S. aureus. However, very little is known about the influence of growth conditions and medium composition on exoprotein production. Moreover, the regulation of the synthesis and the mechanisms of secretion of these exoproteins are generally obscure. One of the exoproteins produced by S. aureus is staphylocoagulase. Although its precise role in the pathogenesis has not been established, staphylocoagulase is still considered as one of the most important virulence factors of pathogenic staphylococci. Furthermore, the ability of staphylococci to produce staphylocoagulase is a major criterion for the identification of S. aureus.

In Chapter 1, a review has been given of the mode of action of staphylocoagulase and its possible roles in the pathogenesis of S. aureus. Since the composition of the cell-wall and the production of exoproteins is strongly influenced by the growth environment of S. aureus, it was of interest to study several aspects of the production of staphylocoagulase in relation to the growth conditions.

The purpose of the investigations presented in this thesis was therefore to assess the influence of medium composition and growth conditions on the production of staphylocoagulase. Furthermore, the mechanism of secretion of this protein was studied by means of various inhibitors. Spontaneous staphylocoagulase-negative mutants were used to study their competitive ability towards their wild type strains and to elucidate the regulation of the synthesis of staphylocoagulase and of exoproteins in general. Because of the enormous capacity of S. aureus to vary phenotypically in response to changes in the growth environment, the majority of growth experiments were performed using a chemostat; in this way the micro-organisms could grow under steady-state conditions in a constant and reproducible environment.

In Chapter 2 the development of a defined growth medium is described in which high yields of staphylocoagulase were obtained from S. aureus strain 104. Besides a simple salts mixture, this medium contained glycerol,

casein hydrolysate and three vitamins. This medium could replace the generally used Brain Heart Infusion medium and also facilitated the purification of staphylocoagulase since it was free of proteins and lipids. Casein hydrolysate could be substituted completely by a mixture of amino acids of an identical composition; removal of any of the amino acids from this mixture reduced the amount of staphylocoagulase. For practical reasons, casein hydrolysate was used in further experiments. It was demonstrated in batch cultures of S. aureus that conditions of oxygen- and magnesium-limitation were required for optimal yields of staphylocoagulase. When the glycerol concentration was raised above 0.2%, a considerable decrease in staphylocoagulase production took place, probably due to a decrease of the pH value of the culture. Therefore a practically constant pH (about 7.4) was another important factor in optimal staphylocoagulase production.

In Chapter 3, the production of staphylocoagulase was studied with continuous cultures of various S. aureus strains in the newly developed medium. One of the major problems in these studies was to minimize excessive foaming. Therefore, the baffles had to be removed from the culture vessel, the impeller speed kept at moderate values and air flushed over the surface. Particular attention was given to the type of antifoam to be used, since occasionally inhibitory effects on the staphylocoagulase production were observed. It was confirmed, that also in continuous culture, conditions of low oxygen availability and magnesium-limitation were required for optimal staphylocoagulase production. The specific rate of the production of staphylocoagulase was dependent on the growth rate. In two bovine strains, the production rate pattern was similar to that of an inducible enzyme sensitive to catabolite repression, although no specific inducer or repressor could be demonstrated. The human strain 104 on the other hand, produced staphylocoagulase constitutively. In all strains the specific rate of production of total extracellular protein was strictly proportional to the growth rate. These experiments clearly showed that the production of optimal amounts of staphylocoagulase was not always related to maximal production of biomass and total extracellular protein. Mannitol functioned as an energy source rather than as a carbon source because it was converted for a major part to acetate and for a minor part to lactate and not to new cell material. It could be demonstrated that the energy state of the cell is also of great importance in

the regulation of staphylocoagulase production. Catabolite repression of staphylocoagulase production by mannitol was not observed in chemostat cultures of S. aureus whereas in batch cultures a considerably inhibition of the staphylocoagulase production by mannitol occurred, indicating that the inhibitory effects of carbohydrates on the production of staphylocoagulase in batch cultures are a consequence of the interaction of the organisms with their environment in a closed system. The bovine strains produced six times more staphylocoagulase in chemostat culture as compared with batch cultures of the same organism. This indicates that chemostat culture by virtue of its ability to provide constant and specific environmental conditions may prove to be a better culture technique for the production of staphylocoagulase.

A cell-bound staphylocoagulase could be detected in chemostat cultures of S. aureus 104 under magnesium- and oxygen-limited growth conditions (Chapter 4). A distribution study revealed that 81% of this enzyme was membrane-bound and could be optimally released by Triton X-100. The remaining part was located in the periplasmic space and was released during protoplasting of the organism. The effects of cerulenin, quinacrine and lincomycin on the production of cell-bound and extracellular staphylocoagulase indicated that the hydrophobic cell-bound form was a precursor in the production of extracellular staphylocoagulase. When magnesium was added to the medium in large excess of the growth requirement, both the production of extracellular staphylocoagulase and the production of cell-bound staphylocoagulase was strongly inhibited to the same extent. Since magnesium influences the membrane phospholipid composition (particularly the content of positively charged lipids) in Gram-positive bacteria, it was concluded that the negative effect of magnesium on the production of extracellular staphylocoagulase was due to a decreased membrane permeability towards cell-bound staphylocoagulase, resulting in a diminished secretion of the protein. The precise mechanism of the secretion of staphylocoagulase could not be established since no detailed characterization of the cell-bound staphylocoagulase was performed. The involvement of a lipid intermediate/exoprotein releasing protease system could not be excluded; however, the effects of cerulenin and quinacrine allowed also another interpretation since no effect of these substances on the production of total extracellular protein was observed. Therefore, before any conclusion

can be drawn about the mechanism of the secretion system of staphylocoagulase and of exoproteins in general, a complete characterization of the cell-bound precursors and releasing protease(s) is essential, especially when signal-peptides are involved.

Cultivation of S. aureus strains 104 and NCTC 8178 in continuous culture gave rise to staphylocoagulase-negative cells which accumulated progressively by virtue of an apparent growth advantage of these staphylocoagulase-negative cells under the growth conditions used (Chapter 5). The loss of production of staphylocoagulase was accompanied by a simultaneous loss of the production of α -haemolysin and leucocidin. Characterization of the strains revealed no further differences in biotype, exoenzymes, phage pattern and plasmid content. From competition experiments cultures at different dilution rates and cultivation temperatures, it was concluded that the accumulation of staphylocoagulase-negative mutants is based on differences in affinity for the limiting substrates rather than on differences in the production rates of total extracellular protein. Moreover, it was demonstrated that the mode of regulation of the production of exoproteins in S. aureus is incompatible with the generally proposed models. In connection with evidence presented in the literature, it was concluded that the observed mutation in these S. aureus strains was due probably to a transposable genetic element rather than to the loss of a plasmid or a prophage. Determination of the LD₅₀ value of these S. aureus strains by intracerebral injection into neonatal mice showed that the mutant strain of strain 104 was significantly less virulent than the parent strain whereas differences in virulence between parent and mutant of strain NCTC 8178 could not be demonstrated.

Chapter 6 describes a rapid and direct assay of staphylocoagulase for the routine identification of S. aureus. The assay is based on the limited proteolysis of the chromogenic substrate Chromozym TH by staphylo-thrombin. The great advantage of this method is that false-positive and false-negative reactions can be eliminated. A comparison of this method with three clotting assays has been made using recently isolated clinical strains of S. aureus. The new assay is specific, quantitative and easily automated. Therefore, it offers clinical laboratories a more reliable assay of staphylocoagulase and it enables an identification of S. aureus within one day. Furthermore, this assay method can be applied excellently for the typing of S. aureus

strains according to the eight different serotypes of staphylocoagulase, described by Dekio and Onimura.

The experiments described in this thesis demonstrate a close relationship between the production of staphylocoagulase by S. aureus and growth conditions. In addition, the secretion process of staphylocoagulase is influenced also by the growth environment. The data presented here are in accordance with present knowledge of the interaction between micro-organisms and their environment, and again demonstrate that the phenotypic variability of micro-organisms has far-reaching consequences for present-day research in medical microbiology. Therefore, data obtained with undefined cultures in undefined growth environments will have a very limited predictive value for the in vivo situation. Although the number of growth conditions studied in this thesis is limited, the results illustrate the importance of the growth environment on the production of staphylocoagulase by S. aureus. It is obvious that much more information is needed about the nature of the growth environment of S. aureus during the infection process. When more data are available about the natural environment of S. aureus which presumably will be severely nutrient-limited, a better insight can be obtained into the role of staphylocoagulase in the virulence and pathogenicity of this organism. It can be expected that investigations into these environmental parameters will provide a better strategy for the treatment and prevention of staphylococcal infections.

SAMENVATTING

Staphylococcus aureus is een commensaal micro-organisme met een lage virulentie. Het veroorzaakt alleen dan ernstige infecties wanneer de normale verdedigingsmechanismen van de gastheer zijn verminderd. De acute ontstekingsreactie en de activiteit van fagocyterende cellen vormen de belangrijkste verdediging tegen stafylococcen-infecties. Anderzijds beschikt S. aureus over een verscheidenheid van virulentie factoren zoals celwand componenten en extracellulaire eiwitten die sterk interfereren met de verdedigingsmechanismen van de gastheer. Hoewel gedurende de laatste jaren onze kennis ten aanzien van de interacties tussen antigene structuur, opsoninen en fagocyterende cellen aanzienlijk is toegenomen, is de rol van extracellulaire eiwitten in de pathogenese van S. aureus nog altijd onduidelijk. Eén van de belangrijkste redenen hiervoor is het ontbreken van een geschikt proefdiermodel.

Sedert inmiddels overtuigend is aangetoond dat veranderingen in de groei-omstandigheden van S. aureus belangrijke veranderingen induceren in antigene structuur, productie van exoeiwitten en biochemische eigenschappen van S. aureus, ten gevolge van fenotypische variabiliteit, wordt men zich steeds meer bewust van het feit dat de virulentie van S. aureus varieert overeenkomstig het milieu waarin de stafylococcen groeien.

In de afgelopen decennia is veel aandacht geschonken aan de productie en karakterisering van exoeiwitten van S. aureus. Er is echter weinig bekend over de invloed van de groei-condities en medium-samenstelling op de productie van deze exoeiwitten. Bovendien is het in het algemeen onduidelijk hoe de regulatie van de synthese en het uitscheidingsmechanisme van exoeiwitten plaats vindt. Eén van de exoeiwitten, geproduceerd door S. aureus, is stafylocoagulase. Alhoewel een rol in de pathogenese niet is vastgesteld, wordt stafylocoagulase nog steeds beschouwd als één van de belangrijkste virulentie factoren van pathogene stafylococcen. Anderzijds is het stafylocoagulase-producerend vermogen van stafylococcen een belangrijk criterium voor de identificatie van S. aureus.

In Hoofdstuk 1 wordt een overzicht gegeven over het werkingsmechanisme van stafylocoagulase en de mogelijke rollen in de pathogenese van S. aureus. Omdat de samenstelling van de celwand en de productie van exoeiwitten sterk wordt beïnvloed door de groei-omstandigheden van S. aureus, was het interessant diverse aspecten van de

roersnelheid terug te brengen tot lagere waarden en lucht over het cultuur-oppervlak te leiden in plaats van door de cultuur. Bijzondere aandacht werd geschonken aan het type antischuim-middel dat moest worden gebruikt omdat geregeld remmende effecten hiervan op de stafylocoagulase productie werden waargenomen. Ook onder continue cultuur omstandigheden bleek een lage beschikbaarheid van zuurstof en een magnesium-limitatie een vereiste voor optimale stafylocoagulase productie. De specifieke productie-snelheid van stafylocoagulase was sterk afhankelijk van de groeisnelheid. In twee runderstammen was het productiepatroon identiek aan dat van een induceerbaar enzym, gevoelig voor kataboliet repressie, hoewel geen specifieke inductor of repressor kon worden aangetoond. Anderzijds produceerde de humane stam 104 stafylocoagulase constitutief. In alle stammen was de specifieke productie-snelheid van totaal extracellulair eiwit strikt evenredig met de groeisnelheid. Deze experimenten lieten duidelijk zien dat de productie van optimale hoeveelheden stafylocoagulase niet altijd gekoppeld was aan een maximale productie van biomassa en totaal extracellulair eiwit. Mannitol functioneerde meer als energiebron dan als koolstofbron omdat het grotendeels werd omgezet in acetaat en voor een klein gedeelte in lactaat, en niet in nieuw celmateriaal. Ook de energie status van de cel bleek van groot belang voor de regulatie van de stafylocoagulase productie. Kataboliet repressie van de stafylocoagulase productie door mannitol werd niet waargenomen in chemostaat culturen van S. aureus terwijl in batch culturen een aanzienlijke remming van de stafylocoagulase productie door mannitol optrad. Dit wijst erop dat de remmende effecten van koolhydraten op de productie van stafylocoagulase in batch culturen het gevolg waren van een interactie van de organismen met hun omgeving in een gesloten systeem. De runderstammen produceerden zes keer zoveel stafylocoagulase in chemostaat culturen dan in batch culturen van hetzelfde organisme. Dit geeft aan dat chemostaat culturen, door hun eigenschap constante en specifieke milieu-omstandigheden te creëren, een betere kweektechniek kunnen zijn voor de productie van stafylocoagulase.

Een cel-gebonden stafylocoagulase kon worden aangetoond in chemostaat culturen van S. aureus 104 onder magnesium- en zuurstof-beperkende groei-omstandigheden. Een verdelingsstudie toonde aan dat 81% van dit enzym membraan-gebonden was en optimaal kon worden vrijgemaakt met behulp van Triton X-100. Het resterend gedeelte was gelocaliseerd in de periplasmatische ruimte en kwam vrij

productie van stafylocoagulase te bestuderen in relatie tot de groei-omstandigheden.

Het doel van het onderzoek, beschreven in dit proefschrift, was daarom na te gaan welke de invloed is van medium-samenstelling en groei-omstandigheden op de productie van stafylocoagulase. Bovendien werd het uitscheidingsmechanisme van stafylocoagulase bestudeerd met behulp van diverse remmers. Spontane stafylocoagulase-negatieve mutanten werden gebruikt om hun competitief vermogen ten opzichte van de wilde stammen te bestuderen en de regulatie van de synthese op te helderen van stafylocoagulase en van exoeiwitten in het algemeen. Omdat S. aureus een zeer groot vermogen bezit om fenotypisch te variëren als reactie op veranderingen in het milieu, werd het merendeel der groei-experimenten uitgevoerd in een chemostaat (continue cultuur); op deze wijze konden de micro-organismen groeien in een dynamisch evenwicht in een constant en reproduceerbaar milieu.

In Hoofdstuk 2 wordt de ontwikkeling van een gedefinieerd kweekmedium beschreven waarin hoge opbrengsten van stafylocoagulase, geproduceerd door S. aureus stam 104, werden verkregen. Dit medium bevatte een mengsel van minerale zouten, glycerol, caseïne hydrolysaat en drie vitamines. Het bleek dat dit medium het veel gebruikte Brain Heart Infusion medium geheel kon vervangen; de zuivering van stafylocoagulase werd hierdoor vergemakkelijkt omdat het nieuwe medium geen eiwitten en lipiden bevatte. Het caseïne hydrolysaat kon op zijn beurt geheel worden vervangen door een mengsel van aminozuren in een identieke samenstelling; wanneer echter één van de aminozuren in dit mengsel werd weggelaten, verminderde de opbrengst aan stafylocoagulase. Uit praktische overwegingen werd het caseïne hydrolysaat gebruikt in de overige experimenten. In zogenaamde batch culturen van S. aureus bleek dat kweekomstandigheden, waarin zuurstof en magnesium beperkend waren, vereist waren voor een optimale stafylocoagulase productie. Glycerol concentraties hoger dan 0,2% remden de stafylocoagulase productie sterk, waarschijnlijk door een daling van de pH van de cultuur. Daarom was een nagenoeg constante pH (ongeveer 7,4) een andere belangrijke factor voor een optimale productie van stafylocoagulase.

In Hoofdstuk 3 werd de productie van stafylocoagulase bestudeerd in continue culturen van diverse S. aureus stammen in het nieuw ontwikkelde medium. Eén van de belangrijkste problemen in deze studies was het verminderen van overmatige schuimvorming. Daarom was het nodig de keerschotten te verwijderen uit het kweekvat, de

tijdens de bereiding van protoplasten van het organisme. De effecten van cerulenine, quinacrine en lincomycine op de productie van cel-gebonden en extracellulair stafylocoagulase gaven aan dat de hydrofobe cel-gebonden vorm een precursor was in de productie van extracellulair stafylocoagulase. Indien magnesium in overmaat aan het medium werd toegevoegd, werd de productie van cel-gebonden en extracellulair stafylocoagulase in gelijke mate sterk geremd. Omdat magnesium de fosfolipide samenstelling (met name het gehalte aan positief geladen lipiden) van de membraan in gram-positieve bacteriën beïnvloedt, werd geconcludeerd dat het negatieve effect van magnesium op de productie van extracellulair stafylocoagulase werd veroorzaakt door een afname in de membraan permeabiliteit voor cel-gebonden stafylocoagulase zodat de uitscheiding van het eiwit werd verminderd. Het juiste uitscheidingsmechanisme van stafylocoagulase kon niet worden vastgesteld omdat geen gedetailleerde karakterisering van het cel-gebonden stafylocoagulase werd uitgevoerd. De rol van een lipid intermediair/exoeiwit vrijmakend protease systeem kon niet worden uitgesloten hoewel de effecten van cerulenine en quinacrine ook op een andere manier konden worden geïnterpreteerd, mede gezien het feit dat geen van deze verbindingen een invloed hadden op de productie van totaal extracellulair eiwit. Voordat enige conclusie omtrent het uitscheidingsmechanisme van stafylocoagulase en dat van exoeiwitten in het algemeen kan worden getrokken, is een complete karakterisering van de cel-gebonden precursors noodzakelijk, vooral wanneer ook nog signaal-peptiden in dit proces zijn betrokken.

Tijdens het kweken van S. aureus stam 104 en stam NCTC 8178 in continue cultuur, verschenen stafylocoagulase-negatieve cellen die konden worden waargenomen ten gevolge van een groei-voordeel van deze stafylocoagulase-negatieve cellen onder de gebruikte kweekomstandigheden (Hoofdstuk 5). Het verlies van stafylocoagulase productie ging gepaard met een gelijktijdig verlies van de productie van α -haemolysine en leucocidine. Een karakterisering van deze stammen liet zien dat er geen verdere verschillen waren in biotype, exoenzymen, faagpatroon en plasmid-gehalte. Uit competitie experimenten bij verschillende verdunnings-snelheden en kweektemperaturen, werd geconcludeerd dat de ophoping van de stafylocoagulase-negatieve mutanten het gevolg was van verschillen in affiniteit voor de groei-beperkende substraten en niet het gevolg van verschillen in de productie-snelheid van totaal extracellulair eiwit.

Bovendien werd vastgesteld dat de regulatie van de productie van exoeiwitten onverenigbaar was met de algemeen voorgestelde modellen. Met behulp van literatuurgegevens werd geconcludeerd dat de opgetreden mutatie in deze S. aureus stammen vermoedelijk werd veroorzaakt door een transposon en niet door het verlies van een plasmid of een profaag. Bepaling van de LD₅₀ waarde van de S. aureus stammen door middel van intracerebrale injectie in neonatale muizen liet zien dat de mutant van stam 104 significant minder virulent was dan de wilde stam terwijl geen verschillen in virulentie tussen de wilde stam en de mutant van stam NCTC 8178 waren aan te tonen.

Hoofdstuk 6 beschrijft een snelle en directe bepalingmethode van stafylocoagulase ten behoeve van de routine identificatie van S. aureus. De bepalingmethode is gebaseerd op een beperkte proteolyse van het chromogene substraat Chromozym TH door stafylothrombine. Het grote voordeel van deze methode is dat vals-positieve en -negatieve reacties kunnen worden geëlimineerd. Een vergelijking van deze methode met drie stollingsmethoden werd gemaakt door gebruik te maken van recent geïsoleerde klinische S. aureus stammen. De nieuwe methode is specifiek, kwantitatief en gemakkelijk te automatiseren. Daarom biedt deze methode het klinisch laboratorium een betrouwbare bepalingmethode van stafylocoagulase en het maakt een identificatie van S. aureus binnen één dag mogelijk. Bovendien is de methode uitstekend te gebruiken voor het typeren van S. aureus stammen volgens de acht verschillende serotypen van stafylocoagulase zoals beschreven door Dekio en Onimura.

De experimenten beschreven in dit proefschrift laten zien dat er een nauwe relatie bestaat tussen de productie van stafylocoagulase door S. aureus en de groeiomstandigheden. Bovendien wordt ook het uitscheidingsproces van stafylocoagulase beïnvloed door het milieu. De weergegeven resultaten zijn in overeenstemming met de huidige kennis over de wisselwerking tussen micro-organismen en hun milieu en laten zien dat de fenotypische variabiliteit van micro-organismen verstrekkende gevolgen heeft voor het onderzoek op het gebied van de medische microbiologie heden ten dage. Daarom hebben gegevens, verkregen met ongedefinieerde culturen in ongedefinieerde groei-omstandigheden, een zeer beperkte voorspellende waarde voor de situatie in vivo. Hoewel het aantal groei-omstandigheden, onderzocht in dit proefschrift beperkt was, illustreren de resultaten het belang van het milieu op de productie van stafylocoagulase door S. aureus. Het is duidelijk dat veel meer

gegevens nodig zijn over de aard van de groei-omstandigheden van S. aureus tijdens het infectie-proces. Wanneer meer feitenmateriaal beschikbaar is over het natuurlijke milieu van S. aureus, dat ongetwijfeld sterk nutriënt-beperkend zal zijn, kan een beter inzicht worden verkregen in de rol van stafylocoagulase in de virulentie en pathogeniteit van dit organisme. Het is te verwachten dat onderzoek naar deze milieu parameters een betere strategie zullen verschaffen voor de behandeling en voorkoming van stafylococce infecties.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 11 november 1946 te Helmond. Het eindexamen HBS-B werd behaald in 1964 aan het Comenius-lyceum te Amsterdam. Daarna volgde hij de studie in de scheikunde aan de Gemeente Universiteit te Amsterdam, waarbij hij in 1971 het doctoraal examen biochemie aflegde. Aansluitend vervulde hij de militaire dienstplicht, waarbij hij werd geplaatst bij de afdeling Bacteriologie van het Rijks Instituut voor de Volksgezondheid (RIV) te Bilthoven. Van 1973-1975 was hij als wetenschappelijk medewerker verbonden aan het laboratorium voor Medische Microbiologie van de Erasmus Universiteit te Rotterdam. Sinds 1975 is de auteur als wetenschappelijk medewerker verbonden aan de capaciteitsgroep Medische Microbiologie van de Rijksuniversiteit Limburg te Maastricht. Onder leiding van Prof.Dr. C.P.A. van Boven werd het onderzoek verricht, hetwelk geleid heeft tot de bewerking van dit proefschrift.