BACTERIOCINS OF STAPHYLOCOCCI AND ORAL STREPTOCOCCI

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF.DR. A.J.H. VENDRIK VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 2 SEPTEMBER 1977 DES NAMIDDAGS OM 2 UUR PRECIES

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Aan mijn ouders

Voor Jos en
Floortje

C O N T E N T S

CHAPTER 1

GENERAL INTRODUCTION

The observation of antagonistic interactions between microorganisms was first recorded in 1877 by Pasteur and Joubert (82). The antagonisms between closely related bacteria were studied by many authors in the late nineteenth century and the early years of this century (see 28,29). Many of the relations found at that time might have concerned bacteriocins, but no definite conclusions can be drawn, since the investigators were more engaged in the biological and therapeutical implications than in the nature of the interacting agents. Progress in understanding the nature of bacteriocins stems from the work of Gratia(33,34) who studied the antagonistic substances of *Escherichia coli* strain ϕ and clearly differentiated bacteriocins from the just discovered phenomena of lytic bacteriophages and lysogeny. Jacob *et al* (46) defined bacteriocins first as bactericidal proteins, produced by lethal biosynthesis, with a narrow spectrum of activity centered about the homologous species and which attach to specific receptors on the surface of susceptible cells. A plasmid-borne genetic determinant of bacteriocin-production and host cell immunity to bacteriocins were later on added to the definition. However, both the original and extended definitions are based on the properties of the bacteriocins of *E. coli*, the colicins. The more recently evolving, still increasing stream of information on bacteriocin-like substances, especially on those of gram-positive bacteria, disputes the original definition since only relatively few of these substances fit well in it. Tagg *et al* (109), in a recent review on bacteriocins of gram-positive bacteria, touched upon the need of a wider concept of bacteriocinogeny. In practice, many investigators have used the term bacteriocin to design bacterial substances with a biologically active protein moiety and bactericidal mode of action.

Various aspects of bacteriocins have been reviewed (14,43,75,90) and monographed (8,89) in the last decade. Recent reviews dealt primarily with the mode of action of colicins (45,69,104), bac-

teriocinogeny from an evolutionary point of view (41) and bacteriocins of gram-positive bacteria (109). They emphasize the invaluable contribution of the study of bacteriocins to extend our insight in various fields as membrane biology, active transport, energy metabolism, macromolecular syntheses, DNA-replication, gene function and evolution.
This introduction will briefly describe recent developments in the investigation of bacteriocins, with special reference to those of staphylococci and streptococci. Some more detailed discussion will be presented with the topics concerned in the subsequent chapters.

Genetics

Extensive studies established the extra-chromosomal character of colicin production (Col factors), most of them existing in an autonomous state (plasmid). Col factors seem to appear in two groups (40) on basis of genetical, physiological and biochemical properties, such as molecular weight, self-transmissibility by conjugation, number of copies per cell, dependency on host functions required for maintenance, and activity of the respective colicins on specific classes of tolerant mutants (41). The mode of action of the colicins is not subject to this classification. Both groups are proposed to be arisen through the divergence from a distinct ancestor, or from the convergence of plasmids of diverse origins to give each of the groups (41).
Not only the chemical nature of the bacteriocin, but also the regulation of its biosynthesis and release from the cell, and the host cell immunity may be determined by the bacteriocinogenic factor. Host cell immunity is quite distinct from bacteriocin resistance, which is determined by the absence of specific bacteriocin receptors on the cell surface. Detailed studies of host cell immunity to colicin E3 (48,102) and cloacin DF13 (24, 61), both inhibiting protein synthesis, have shown that a 1:1 stoichiometric complex between the active bacteriocin and an immunity protein is formed, resulting in an inactivation of the first one. Recently, an immunity protein has been found to be associated also with colicin E2 (99). At high bacteriocin concentrations immunity may be incomplete (67) as was also re-

ported for bacteriocins of staphylococci (31) and streptococci (62,107). The nature of immunity is yet poorly understood in these bacteria but may be analogous to the colicin system. Bacteriocinogenic strains of *S. faecalis* var. *zymogenes* seem to be protected by the possesion of a specific teichoic acid (20). The establishment of the plasmid-borne nature of bacteriocinogeny in gram-positive bacteria is mainly based on indirect evidence of spontaneous or curing agents-induced loss of this property, which has been observed in staphylococci (31,51,65,71, 114) and streptococci (64,111), but suffers serious limitations (106). Only recently it was shown that staphylococcin R_1 of phage group II *Staphylococcus* was determined by a large 56S virulence plasmid (93,115) and the plasmids determining the bacteriocins(hemolysins) of *S. faecalis* var. *zymogenes* (47) and *S. faecalis* 5952 (78) were characterized. Transmission of bacteriocinogenic factors by conjugation between streptococci has been described (47,110), and transduction was applied to transfer the bacteriocinogenic factor in staphylococci (113). Sensitivity to a bacteriocin produced by *S. faecalis* 5952 was conferred by the presence of a plasmid in another strain of *S. faecalis* (77).

Mode of action

Classification of bacteriocins according to their mode of action is based on the types of biochemical lesions observed upon the action of various colicins (75), which allowed the division into three classes.

The first class, represented by colicins E1 and K (see 69; 11, 85), Ia and Ib (32,67,73), A (21,72) and a number of less well-investigated colicins, is characterized by a simultaneous, independent inhibition of the syntheses of DNA, RNA, protein and polysaccharide -but not accompanied by an extensive degradation of the macromolecules-, a rapid arrest of active uptake coupled to a high-energy state of the membrane and abolition of the electrical potential of the membrane. Leak of electrolytes and decrease of cellular ATP level are observed, but no gross impairment of the permeability barrier, proton permeability or respiration. The decrease of ATP level was shown to be attribu-

table to the attempt of cells to reenergize the membrane (27, 85). These cell functions are thought to be affected through an effect on the cell membrane, on basis of the results of direct observations of the effect of colicin E1 (5) and A (52) on transport in isolated membrane vesicles. The first prove of a distinct membrane defect induced by colicins was delivered by Sabet (95,96) who showed that the ATP-linked transhydrogenase reaction was impaired in membranes prepared from bacteria treated with colicin E1 or K. Knepper and Lusk (57) showed that specific proteins had disappeared from the membranes of cells treated with colicin E1 or K. Most promising appear experiments with mutant strains resistant to colicins of this class and simultaneously affected in parts of the energy metabolism (59,68, 86). The *ecf* gene product may be the target of colicin K (45a). Many of the bacteriocins of gram-positive bacteria appear to belong to this class, since they clearly affect the permeability of the cell membrane. The most thoroughly investigated bacteriocin of this group is staphylococcin 1580 (50,52,53, this thesis), which shares many of the properties outlined above. Similar effects have been observed with staphylococcins C55 (16,18) and 462 (36) -although the latter is said to have only a bacteriostatic effect-, streptococcins A-FF22 (107) and STH_1 (100) and the enterocins E1A and E1B (63).

The model of the second class of bacteriocins is colicin E2, which exerts its most conspicuous effect on the degradation and solubilization of DNA (74). Although a role of endonuclease I in the colicin E2 action has been proposed (1,2), recent experiments showed that the purest preparations of colicin E2 introduced one single-strand scission in supercoiled λ phage DNA (98). Schaller and Nomura (99) have recently shown that colicin E2 preparations contain a low molecular weight immunity protein and that preparations freed from the immunity protein showed a high *in vitro* activity in cleaving various DNA molecules.

In addition to the effect on DNA, colicin E2 inhibits active transport in *E. coli* cells which carry the *rex* gene of λ prophage (4,97). Colicin E2 also acts against the flagellate *Euglena gracilis* (105). The only bacteriocin of gram-positive bacteria thus far claimed to exert an effect like colicin E2 is

megacin C-C4A$^-$ (44), produced by *Bacillus megaterium*.
The third class of bacteriocins is represented by colicin E3 and cloacin DF13, of which the mode of action has been elucidated into detail. It was shown that both *in vivo* (7,23,101) and *in vitro* (6,23) 16S ribosomal RNA was cleaved at an unique site near its 3' end. In addition, cloacin DF13 induces permeability changes in the cell membrane (22).
Recent reports indicate that a bacteriocin of *Lactobacillus helveticus*, named lactocin LP27, affects susceptible bacteria in a way comparable to the effect of cloacin DF13 (112). Also megacin Cx inhibits primarily protein synthesis (26).
Various agents produced by gram-positive bacteria and claimed to be bacteriocin-like, cannot be classified according to the above scheme. They bring about various phenomena such as bacteriolysis, bacteriostasis, sporostasis, and spheroplast formation (109).
The action of colicins is believed to occur in at least two successive stages (83). The first stage represents binding of the bacteriocin to the specific surface receptor, without the induction of physiological injury. During this stage the cells may be rescued by inactivation of the bacteriocin by trypsin (83), antisera (70) or sodium dodecyl sulfate (13); however, the rescue has recently been questionned by Cavard (13). The transition to stage II, causing specific biochemical lesions, is dependent on temperature and the fatty acid composition of the membrane (76,84) and is blocked by inhibitors of the energy metabolism (84,91,92). Transition to stage II is presumably attended with the movement of the colicin, or colicin-induced substances through fatty acid-containing regions of the cell envelope and with changes in the microviscosity of the cell envelope (15,42,73). An energized state of the membrane appears to be required for the transmission process (54). It is of interest whether a similar scheme is applicable to interactions of other agents with susceptible cells, since energy requirement was noted in the antibody-complement killing of *Pasteurella septica* (35) and the action of basic proteins on *Candida albicans* (79).
The existence of specific bacteriocin receptors in the cell en-

velope has been well established in case of the colicins. Resistance due to the lack of specific receptors formed the basis of Fredericq's classification of the colicins (30). The receptors for colicin E3-CA38 (94), colicin M-K12 (9) and colicins Ia-CA53 and Ib-P9 (58) have been isolated and purified. Bacteriocin receptors are often shared by bacteriophages. An intriguing observation, considering the evolutionary development of bacteriocin receptors, was the identification of the colicin E receptor as the receptor for vitamin B12 (25,55). Recent interest focussed on the role of the receptors for colicins of the B-group (B,D,G, H,Ia,Ib,M,Q,S1 and V) in the uptake of iron complexed with either citrate, enterochelin or ferrichrome (10,37,60,87). The colicin K-phage T6 receptor is presumably involved in the uptake of nucleosides (38).

Although adsorption was shown to be highly specific for susceptible bacteria in the case of staphylococcin C55 (17), the adsorption appears to be aspecific for many other bacteriocins of gram-positive bacteria, such as staphylococcin 1580 (49) and 414 (31), lactocin LP27 (112) and streptococcin B-74628 (108). Others, like viridin B (19) and presumably staphylococcin 462 (36), do not adsorb at all to susceptible bacteria. Since the cell wall of many gram-positive bacteria allows the passage of relatively large molecules such as extra-cellular enzymes, the function of receptors in these bacteria may be different from those found in the outer membrane of *E. coli*. The cell wall may protect resistant mutants against the action of staphylococcin 1580 (53), although the production of extra-cellular enzyme appears not to be affected (Weerkamp, unpublished observation).

Ecology

It is evident that a selective advantage has led to the evolution and survival of the capability to produce bacteriocin. At the present state of knowledge it is not possible to assess if the bacteriocinogenic clone, the bacteriocinogenic factor or the gene coding for the bacteriocin is selected (41). Hardy (41) proposed the importance of bacteriocinogeny in increasing the chance of survival of the bacteriocinogenic factor by enabling its host to kill equally competitive bacteria. In the presence

of a more competitive strain the factor might be transferred to this dominant one. The possibility that such strategies have evolved might be obvious in view of the important evolutionary role of extra-chromosomal elements, as suggested by Reanney (88). More obscure is the function of the evolutionary development of receptors, unless they fulfill some vital functions (89,90). The involvement of colicin receptors in other cell functions has been mentioned above.

In spite of the well-established and widespread production of bacteriocins in laboratory strains, the action of bacteriocins in nature is apparently less obvious. This contrast is less pronounced in the case of the Enterobacteriaceae, since only a very small part of a clone actually produces the bacteriocin in uninduced cultures (81), although a considerable variation depending on the growth phas may be observed (39). However, many of the bacteriocins of gram-positive bacteria are produced constitutively and by almost all cells of a clone.

Studies on the effect of colicinogeny on the intestinal flora of various kinds of animals revealed that, although the colicinogenic strain often becomes predominant, no effect could be attributed to the production of the colicin, since no differences were found whether sensitive or resistant strains were co-established (see 41), probably due to the inactivation of the colicin in the intestinal canal (56). Similarly, Col V^+ strains exceeded Col V^- organisms in various tissues and body fluids when applied to various kinds of animals, but the effect could not be attributed to killing of the sensitive strain by collicin V, although the colicin could be demonstrated in the blood. At locations remote from the defence mechanism of the body, occasionally the number of Col V^- organisms even exceeded those of Col V^+ (116). The virulence of bacteriocinogenic strains may often be explained by the association with pathogeny or drug resistance, such as established for phage group II staphylococci (93) and for *Pasteurella pestis* (12).

The use of avirulent, inhibitory strains to prevent the establishment of, or to displace pathogens has been practized before antibiotics were used as therapeutics (28,29), and has received renewed interest as ecologically more valid means of

pathogen control. *S. aureus* 502A has been succesfully used to control outbreaks of staphylococcal infections and to treat patients with recurrent furunculosis (3,103). The nature of this inhibitory phenomenon has not yet been established.
Staphylococcin A-1262a has been used by Lachowicz to treat a variety of staphylococcal lesions (65).
Relatively much efforts have been undertaken to establish a possible role for bacteriocins in regulating the dental plaque flora. These studies will be discussed in Chapters 2 and 3.

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CHAPTER 2

BACTERIOCINS AS FACTORS IN THE INTERACTION BETWEEN ORAL STREPTOCOCCI IN *IN VITRO* PLAQUE

INTRODUCTION

The presence of dental plaque on teeth has long been known to be associated with dental caries and periodontal disease. Streptococci are prominent organisms in plaque and in carious lesions (2,3,21). *Streptococcus mutans* is thought to be important in the initiation of carious lesions (8,22,30). Quite strong evidence indicates that adherence of microorganisms is a regulator of plaque flora *in vivo* (10,11,17,23). In addition, the involvement of factors such as nutrient requirements (4), the production of antagonistic substances such as peroxide (7,16), acid (7,9,25) and bacteriocins have been proposed mainly from *in vitro* experiments. Bacteriocins are produced widely by oral streptococci *in vitro* (12,19,28,29) and its production was shown to be a species specific character, with potential significance in dental plaque (31). Wether or not bacteriocins are actually produced *in vivo* is unknown. Although a number of objections against an ecological role of bacteriocins in dental plaque have been raised by some authors (7,20,27), these objections are based on indirect evidence. This paper reports on the production and effect of two bacteriocins on the microbial composition of *in vitro* plaque, formed on glass rods.

MATERIALS AND METHODS

Microorganisms

S. mutans SW31, *S. mutans* P14B4 and *S. sanguis* P3A3 were isolated from human dental plaque. *S. mutans* OMZ61 and *S. sanguis* Ny101 were obtained from Dr. H. van der Hoeven, Institute of Preventive and Community Dentistry, Nijmegen. To facilitate identification in mixed culture, streptomycin-

and erythromycin-resistant mutants (indicated by the suffices s and e, respectively) were selected from some of the strains. The strains were subcultured each fortnight on TY agar, consisting of 3% tryptone soya broth (Oxoid) and 1% yeast extract (Oxoid) solidified with 1.5% agar (Difco), in B&T jars (Searle Comp.) filled with 90% N_2 and 10% CO_2.

Preparation of mutacin SW31

S. mutans SW31 was inoculated from an overnight culture into a medium which consisted of 2% tryptone (Oxoid), 1% Lab Lemco powder (Oxoid), 0.8% sucrose, 0.2% NaCl, 0.2% $NaHCO_c$ and 0.04% Na_2HPO_4, and grown in static culture for 40 hours at 37°C. All subsequent steps were carried out at 4°C. Cells were removed by centrifugation for 15 min at 12.000 x *g*. The clear supernatant was brought to 35% saturation by slowly adding powdered ammonium sulphate under constant stirring. After 60 min the precipitate was pelleted by centrifugation for 20 min at 18.000 x *g*. The pellet was dissolved in one hundreth of the original volume of 0.05 M potassium phosphate buffer (pH 7.0), dialyzed overnight against a large excess of the same buffer and heated for 15 min at 70°C. The resultant preparation could be stored at 4°C without loss in activity for at least two weeks and was used in the experiments. Further purification resulted in highly labile preparations, not suited for the present work.

Determination of properties of mutacin SW31

The apparent molecular weight of mutacin SW31, prepared by ammonium sulfate precipitation, was calculated from its elution pattern of a Sephadex G100 column (1.5 by 45cm) prepared in 0.05 M potassium phosphate buffer (pH 7.0). The column was previously calibrated using Blue Dextran 2000, bovine serum albumin and cytochrome c as standards.

The isoelectric point of mutacin SW31 was determined by use of a LKB 8100-1 Ampholine column with carrier ampholytes of pH 3-10, operated at 14 Watt for 72 h. Fractions were collected and assayed for mutacin activity, protein (absorption at 280 nm) and pH.

The effect of various enzymes on mutacin activity was tested by incubating the bacteriocin (final concentration 250 AU/ml) for 15 min at 37°C with 0.2 mg/ml of the enzymes in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.01 M magnesium sulfate. Subsequently the bacteriocin activity was tested as described below. No inhibition zones were observed in experiments with the enzymes alone.

Bacteriocin activity testing procedures

Production of bacteriocin in agar medium was tested by the stab inoculation technic. Cells of an overnight broth culture were stab inoculated into agar plates and incubated for 48 h at 37°C under N_2/CO_2 atmosphere. After this time overnight cultures of the indicator strain, diluted 1000-fold into fresh medium, were sprayed over the plates, which were incubated for another 24h. Clear zones in which growth was absent appeared around inoculated spots of producer strains.

To establish if an inhibition zone was due to the presence of a bacteriocin, plates were sprayed after the initial growth with a filter-sterelized solution of trypsin (1 mg/ml), incubated for 1 h at 37°C and subsequently sprayed with a solution of trypsin-inhibitor (2 mg/ml). After standing at 37°C for about 1 h to allow the surface of the agar to dry, the plates were sprayed with the indicator strain.

The presence of bacteriocins in broth cultures was tested after removal of the cells by centrifugation for 15 min at 12.000 x *g* 0.1 ml amounts of the supernatant were pipetted into wells (9 mm diameter) punched in TY agar plates, previously seeded with about 10^5 colony forming units of the indicator strain. The plates were subsequently incubated at 37°C for 24 h, after which bacteriocin activity had resulted in clear zones around the wells.

Quantitation of cell-free bacteriocin preparations was carried out by pipetting 0.1 ml amounts of serial twofold dilutions into the wells. *S. sanguis* Ny101 was used as the indicator strain. After overnight incubation the approximate surface area of the inhibition zones was plotted against $\log_2$ of the dilution factor. The apparent dilution at the dissection point of

the resulting straight line with the dilution axis was assumed to contain 1 arbitrary unit (AU) of activity per 0.1 ml. Assay of bacteriocin in plaque samples was carried out by a modification of the well-diffusion test. Rods with adherent plaque were transferred to sterile TH-sucrose broth, which consisted of 3.64% Todd Hewitt broth (Oxoid) supplemented with 2% sucrose, and incubated for another 6 h at 37°C. Subsequently the rods were carefully rinsed with 5 ml saline (0.85% NaCl) and the plaque was scraped into about 0.5 ml soft (0.8%) TY agar at 70°C and kept for 15 min at this temperature. One-tenth ml amounts of plaque suspension were pipetted into wells as described. After overnight incubation clear zones around the wells indicated bacteriocin activity. Alternatively, the rods with adherent plaque were treated with chloroform vapor for 15 min and subsequently treated as described, but omitting the temperature step. Culturing of temperature- or chloroform-treated plaque suspensions showed complete absence of surviving cells.

Determination of the killing effect

S. sanguis Ny101 was grown into mid-exponential phase in TY medium at 37°C in static culture. Cells were centrifuged, washed and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) at a concentration of 8.0×10^8 cells/ml. The suspension was split into two portions and incubated at 37°C. After 5 min, mutacin SW31 was added (final concentration 100 AU/ml) to one portion, the other received only buffer. At the start and the times indicated by an arrow, samples were withdrawn and further incubated with trypsin (0.2 mg/ml). At intervals aliquots were taken from all incubations, rapidly diluted more than 100-fold into icecold TY medium and plated on TY agar.

In vitro plaque formation

The method used was a modification of that described by Jordan and Keyes (18). Plaque was formed on 12 cm long and 0.4 cm wide glass rods suspended from silicone rubber stoppers into 19 cm x 1.5 cm test tubes, filled to 2 cm from the top with TH-sucrose broth. The medium was inoculated with 0.2 ml of an overnight culture grown in TH broth. When mixed-culture plaque

was required, 0.2 ml of each separately grown strain was used. After 24 h at 37°C, rods with adherent plaque were transferred to test tubes filled with freshly inoculated medium. This was repeated daily until the plaque was used for analysis.

Analysis of in vitro plaque

Rods were removed from the medium and carefully dried with tissue. Wet weights of plaque material were measured by weighing rods plus plaque, subsequently scraping off the total plaque substance into 6 ml of sterile saline and reweighing the rods. The bacterial composition was determined after dispersion of the plaque material by two bursts of ultrasonic oscillation for 20 s with a microtipped Branson sonifier at 75 Watt and subsequent culturing of serial dilutions of the suspension on TY agar supplemented with streptomycin or erythromycin (both 0.1 mg/ml) and incubation for 48 h under N_2/CO_2 atmosphere.

Mutagenic procedures

Non-bacteriocinogenic mutants of *S. mutans* SW31 were obtained according to a modified method of Adelberg (1). Cells from 10 ml of a mid-exponential phase culture, growing anaerobically in TH broth, were collected on Millipore filter (0.45 µ), washed and resuspended in an equal volume of 0.05 M tris (hydroxymethyl) aminomethane-maleic acid buffer (pH 6.0) containing 100 µg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NG) and incubated at 37°C for 30 min. Subsequently cells were filtered, washed twice with buffer and resuspended in 50 ml of TH broth. After growth at 37°C for about 4 h, the cell suspension was diluted to give about 50 colonies per plate when plated on TH agar. Plates were incubated anaerobically for 48 h and subsequently sprayed with a suspension of *S. sanguis* Ny101 (about 10^5 colony forming units per plate). Absence of a clear zone around the primary colonies after another 24 h of incubation indicated loss of bacteriocin-producing ability. Such colonies were picked up, purified and further tested for bacteriocin production.

Chemicals

Trypsin (80,000 units/g) and trypsin-inhibitor were obtained

from Merck (Darmstadt, W. Germany), streptomycin from Mycopharm (Delft, The Netherlands), erythromycin lactobionate from Abbott (Brussels, Belgium) and NG from Fluka (Buchs, Switzerland).

RESULTS

Production and properties of mutacin SW31

S. mutans SW31 produces in agar and liquid media an antibacterial substance with the characteristics of a bacteriocin. The highest activity in agar medium was obtained with TH agar; activity in liquid medium was only produced when sucrose was incorporated in the medium. The molecular weight of the mutacin after ammonium sulphate precipitation was about 40.000 daltons as determined from Sephadex-G100 gel filtration and the isoelectric point was about 8.0. In Table 1 some additional properties are given.

TABLE 1. EFFECT OF VARIOUS TREATMENTS ON MUTACIN SW31 ACTIVITY

stable	labile[a]
pH 1-10 (10 min 37°C)[b]	
70°C, 15 min	120°C, 15 min (50% loss)
DNase	Trypsin
RNase	Pronase P
Phospholipase C	Lipase
Phospholipase D	

[a] activity fully destroyed, unless otherwise indicated.

[b] in acetate or phosphate buffer, adjusted to the desired pH and afterwards neutralized with NaOH or HCl.

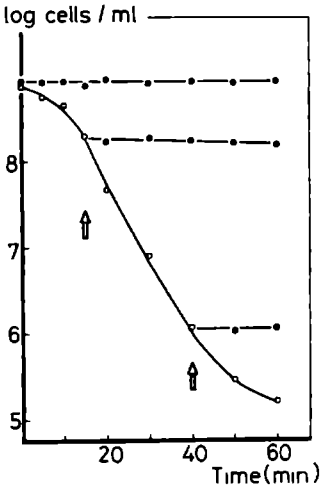

Fig. 1. Killing effect of mutacin SW31
Washed cell suspensions of *S. sanguis* Ny101, containing 8.0 x 10^8 cells/ml in 0.05 M phosphate buffer (pH 7.0), were incubated at 37°C with 100 AU/ml of mutacin SW31 (ammonium sulphate preparation). At the start and times indicated by an arrow samples were withdrawn and further incubated with trypsin (0.2 mg/ml). At intervals samples were rapidly diluted 100-fold into icecold TY broth and subsequently plated in suitable dilutions.
Open symbols: treated with mutacin only, closed symbols: treated with mutacin and subsequently with trypsin.

Sensitive cells are rapidly killed, but may be rescued by the action of trypsin (Fig. 1), which rapidly destroys the bacteriocin and not the bacteriocin receptors presumed to be present in the cell envelope, since cells pretreated with trypsin are not protected from killing (not shown). The action spectrum (Table 2) is characterized by the relatively narrow range of susceptible strains, when compared to other mutacins (13,29); however, it is similar to a *S. mutans* GS5 mutacin described by Paul and Slade (24).

Growth of in vitro plaque

Plaque wet weights increased linearly with time during incubation for several days (Fig. 2,3). However, different strains had quite different rates of plaque formation and the number of colony forming units per weight unit of plaque substance varied significantly and ranged between 10^9 and $6x10^{10}$ per gram wet weight in single strain cultures.
The number of colony forming units per g wet weight increased in time for some strains, while it decreased slightly with

TABLE 2. ACTION SPECTRUM OF MUTACIN SW31

Indicator strain	number of strains tested[a]	number of strains sensitive[a]
Streptococcus mutans (serotypes a-d)	18	0
Streptococcus sanguis	14	12
Streptococcus salivarius	8	7
other streptococci (serogroups A-C, E-G, K-P, R-U and *Streptococcus uberis*)	27	25
Streptococcus faecalis/faecium (serogroup D)	5	0
Actinomyces sp. (oral)	4	2
other gram-positive bacteria[b]	25	0
gram-negative bacteria[c]	19	0

[a] determined with the well-diffusion test as described in the material and methods section.

[b] genera include *Staphylococcus, Bacillus, Lactobacillus, Nocardia, Bacterionema*

[c] genera include *Escherichia, Citrobacter, Fusobacterium, Neisseria, Proteus, Pseudomonas, Serratia, Veillonella*

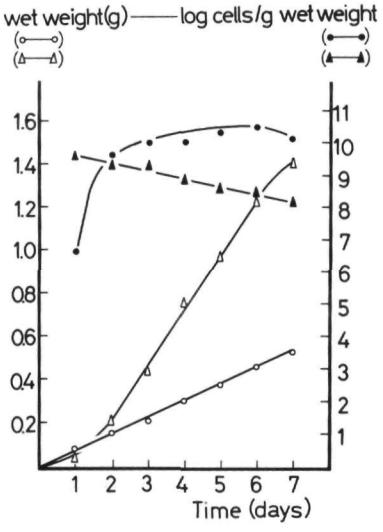

Fig. 2. Growth of single strain plaques on glass rods in TH-sucrose broth.
Open Symbols: wet weight of plaque.
Closed symbols: number of viable cells per g wet weight of plaque.
S. mutans OMZ61 (Δ,▲)
S. mutans P14B4 (0,●).

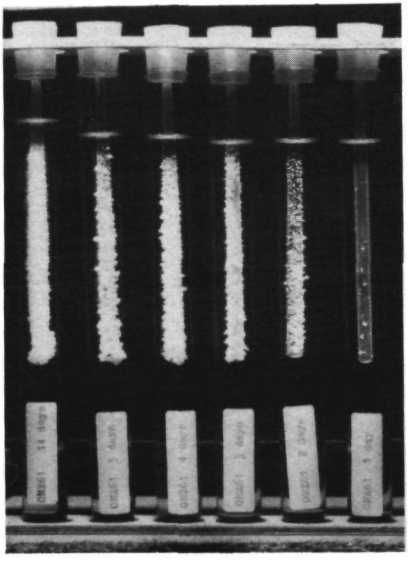

Fig. 3. Growth of *S. mutans* OMZ61 on glass rods **in** TH-sucrose broth.

TABLE 3. ANTAGONISTIC ACTIVITY OF THE STRAINS USED IN THE PLAQUE EXPERIMENTS

strains tested		width of inhibition zone (mm)[a]			
		agar medium test[b]		*in vitro* plaque system[c]	
Producer	Indicator	untreated	trypsin treated	liquid medium	plaque substance
S. mutans SW31	*S. sanguis* Ny101s	10(S)	2(H)	5	5
S. sanguis Ny101s	*S. mutans* SW31	2(H)	2(H)	0	
S. mutans SW31K2	*S. sanguis* Ny101s	2(H)	2(H)	0	0
S. sanguis Ny101s	*S. mutans* SW31K2	2(H)	2(H)	0	
S. sanguis P3A3	*S. mutans* OMZ61s	10(S)	0	0	4
S. mutans OMZ61s	*S. sanguis* P3A3	0		0	0

[a] measured from the edge of the colony or well to the margin of growth.

[b] stab inoculation test with TH-sucrose agar was used. Trypsin treatment was carried out as described in the methods section. (S) refers to zones with clear, sharp edges, (H) indicates hazy zones with diffuse edges.

[c] well diffusion test was used for samples taken from the liquid medium after removal of the cells by centrifugation. Plaque samples were tested in an analogous way, as described in the methods section.
In separate experiments it was shown that the material produced by *S. mutans* SW31 and *S. sanguis* P3A3 was destroyed by trypsin.

others (Fig. 2). This value may be influenced by factors such as the composition and amount of plaque matrix, the proportion of dead cells in the plaque and by the incomplete dispersion of plaque material. The latter was routinely checked for by microscopic observation of plaque suspensions. With the strains used in the present investigation clumping, although not completely absent, was not of major influence on the results.
All *S. mutans* strains used and, in addition, *S. sanguis* P3A3 were plaque formers in single strain cultures.

Production of bacteriocin in plaque
In TH-sucrose agar both *S. mutans* SW31 and *S. sanguis* P3A3 produce large inhibition zones against the indicator strains used (Table 3). After treatment with trypsin no or only small zones were observed. However, *S. mutans* SW31K2, a non-bacteriocinogenic mutant of *S. mutans* SW31 produces only a small, hazy, trypsin resistant zone. Under the conditions used in the plaque experiments mutacin SW31 can be detected in the liquid medium as well as in the plaque substance, whereas the *S. sanguis* P3A3 bacteriocin is detected in the plaque substance only. The mutant strain SW31K2 was non-bacteriocinogenic both in the liquid medium and in the plaque substance.

Influence of mutacin SW31 on mixed plaque composition
Mixed growth of *S. mutans* OMZ61s and *S. sanguis* Ny101e results in plaques with fairly constant proportions of both strains in time. The strains produce no antagonistic substance to each other in plate tests. On addition of mutacin SW31 to the culture liquid the amount of viable *S. mutans* OMZ61s (resistant to the bacteriocin in plate test) remains unchanged, whereas the viability of *S. sanguis* Ny101e (sensitive to mutacin SW31) decreased rapidly to below the limit of detection (Fig. 4).
In control experiments with an identical preparation of the supernatant taken from *S. mutans* SW31K2 cultures only a slight effect on the viability of *S. sanguis* Ny101e was observed (Fig. 4). These results indicate that externally added bacteriocin diffuses into the plaque and remains active long enough to kill the sensitive cells present.

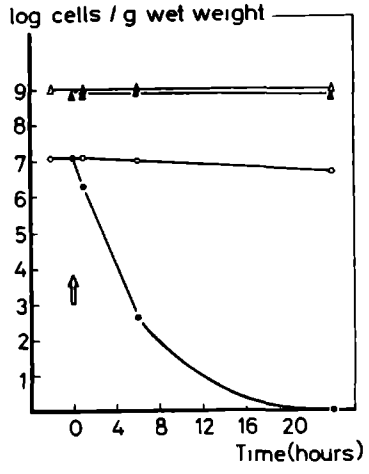

Fig. 4. Influence of mutacin SW31 on mixed plaque of *S. mutans* OMZ61s (Δ,▲) and *S. sanguis* Ny101e (0,●). At the time indicated by the arrow the plaques were transferred to sterile media containing either 30 AU/ml of mutacin SW31 (closed symbols) or an equal amount of a preparation obtained from the non-bacteriocinogenic strain *S. mutans* SW31K2 (open symbols).

TABLE 4. ANALYSIS OF *IN VITRO* PLAQUE AFTER FOUR DAYS OF MIXED CULTURE

Combined strains	cells/g wet weight	ratio	plaque weight (g)[a]
S. mutans SW31	1.8×10^9	2.5×10^6	0.17
S. sanguis Ny101s	7.1×10^2		
S. mutans SW31K2	8.0×10^8	73	0.16
S. sanguis Ny101s	1.1×10^7		
S. mutans SW31	7.4×10^8	1.5	0.17
S. sanguis SW31K2s	4.9×10^8		

[a] plaques formed by single strain cultures of *S. mutans* SW31 and *S. mutans* SW31K2 weighed 0.17 and 0.15 g respectively.

Mixed growth of bacteriocin-producing and sensitive strains

Analysis of plaques after 4 days of mixed growth (Table 4) shows that the bacteriocinogenic *S. mutans* SW31 outnumbers the sensitive *S. sanguis* Ny101s strongly; however, when non-bacteriocinogenic mutant SW31K2 is used, *S. sanguis* Ny101s makes up a substantial part of the viable plaque flora. The pH of the incubated media was identical with both combinations. The results might have been influenced by altered properties of the mutant strain other than the loss of the production of an antagonistic substance: such as growth rate, plaque forming ability or clumping. Therefore the parent and mutant strain were grown together in mixed plaque. Both strains were recovered in almost equal numbers. Furthermore, the amount of plaque wet weight was almost identical for both strains.

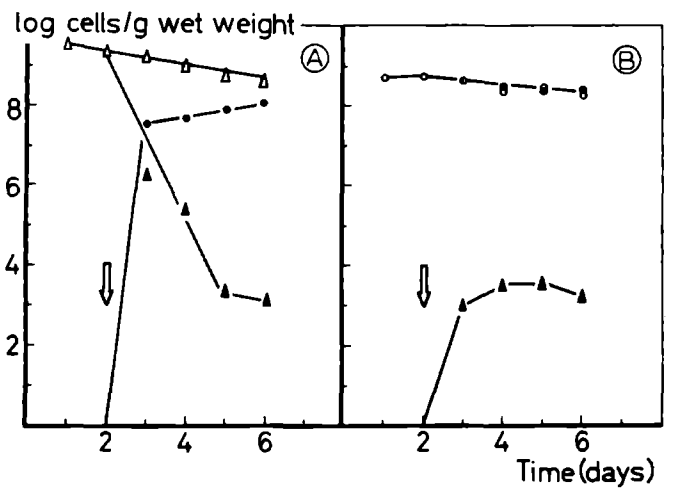

Fig. 5. Effect of superinfection of preestablished plaque with bacteriocin producing and non-producing strains on the bacterial composition of the plaque.
A. Plaque preformed by *S. mutans* OMZ61s (Δ,▲) was incubated with cultures of the bacteriocin producing *S. sanguis* P3A3 (●) at the time indicated by the arrow.
B. *S. sanguis* P3A3 plaque (○,●) was incubated with cultures of *S. mutans* OMZ61s (▲).
Open symbols represent single strain cultures of the strain indicated; closed symbols represent the number of cells in mixed cultures.

TABLE 5. ANALYSIS OF SINGLE STRAIN AND MIXED CULTURES OF *STREPTOCOCCUS MUTANS* OMZ61s AND *STREPTOCOCCUS SANGUIS* P3A3 AFTER FOUR DAYS OF INCUBATION

strains[a]	plaque				medium		
	cells/g wet weight			g wet weight	cells/ml medium		
	S.mutans	*S.sanguis*	ratio[b]		*S.mutans*	*S.sanguis*	ratio[b]
S. mutans OMZ61s	9.0×10^8			0.79	NT		
S. sanguis P3A3		3.2×10^8		1.01		NT	
(P) *S. mutans* OMZ61s	2.5×10^5		1.6×10^2	0.88	$<10^3$	1.6×10^6	$>1.6 \times 10^3$
(S) *S. sanguis* P3A3		4.0×10^7					
(P) *S. sanguis* P3A3		3.3×10^8	1.1×10^5	0.73	4.2×10^5	2.0×10^6	4.8
(S) *S. mutans* OMZ61s	3.1×10^3						

[a] plaques of the primary strains (P) were transferred after two days of growth to media inoculated with the secondary strain (S) and further incubated for two days.

[b] ratio of *S. sanguis* P3A3 over *S. mutans* OMZ61s.

NT = not tested

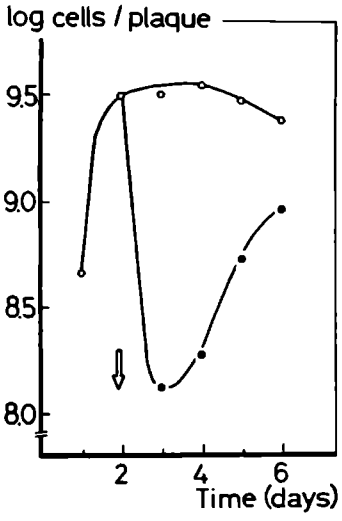

Fig. 6. Total number of viable cells in preestablished *S. mutans* OMZ61s plaque alone (O) and after incubation with *S. sanguis* P3A3 added at the time indicated by the arrow (●). Data were derived from the experiment described in Fig. 5A.

To investigate the role of bacteriocin in the establishment of strains in preexisting plaque, the two plaque-forming strains *S. mutans* OMZ61 and *S. Sanguis* P3A3 were successively inoculated with a two days interval (Fig. 5). *S. mutans* OMZ61 plaque is rapidly invaded by bacteriocinogenic *S. sanguis* P3A3. In the reverse experiment *S. mutans* OMZ61s does not significantly contribute to the total plaque flora, although considerable amounts of *S. mutans* OMZ61s were found in the culture medium (Table 5). Analysis of the total number of viable cells in the plaque (Fig. 6) shows that *S. mutans* OMZ61s is not just overgrown by *S. sanguis* P3A3, but is actually killed, since 24 hours after the addition of *S. sanguis* P3A3 the total viable cell count dropped to only 5% of its original value. The proportion of *S. mutans* OMZ61s at this time is only 0.15% of the original viable count (Fig. 5).

Data sofar obtained concerning the possible role of bacteriocins in microbial ecology are conflicting (15). Particularly this is true for dental plaque, although bacteriocins are produced abundantly by oral streptococci (12,19,28,29). Rogers (26) reported that bacteriocin production depends on specific nutritional requirements which will be met hardly *in vivo*. Since bacteriocins are proteins and thus potentially susceptible to the action of proteases, they would be inactivated rapidly *in situ* by plaque proteases (20). However, according to others (6, 12) many bacteriocin-like substances, although sensitive to trypsin, were only slightly affected by plaque proteases. A third objection was raised as a result of the observation that a protection against bacteriocin action was induced in sensitive oral streptococci on growth in media containing sucrose (27,31), presumably as a consequence of the diffusion barrier provided by the extracellular polysaccharides formed. However, also this phenomenon was not observed with all strains (6,31). The ultimate proof of the active role of bacteriocins in plaque would be delivered by a demonstration of a direct influence of bacteriocins on the plaque ecology.

The present results clearly indicate that bacteriocins are produced in *in vitro* plaque, kill sensitive cells and thus regulate the microbial composition of the plaque.

The plaque matrix does not prevent the action of bacteriocins but instead may serve as a stabilizing environment, as was suggested by the following observations (i) incorporation of sucrose in the medium is required to obtain active mutacin SW31 in liquid culture, but may be replaced by glucose in agar medium, (ii) antagonistic activity could be detected in plaques of *S. sanguis* P3A3 but not in the surrounding medium. The idea is further stressed by the observations made by other investigators (5,24) who reported that mutacin activity was associated with the surface of the producer cells from which it can be dissociated by mechanical treatment.

It is tempting to extend the present findings to natural dental plaques. Arguments against an ecological role for bacteriocins

in dental plaque have been made because bacteriocinogenic and sensitive strains coexist at the same sites (19,27). However, no confirmatory data were presented by the authors. In this connection it is of interest that the mutual inhibition of streptococcal strains in small plaque samples is somewhat lower than among strains each isolated from plaques obtained from different toothsurfaces (31). Histological investigation of plaque shows that bacteria often exist as discrete microcolonies (14). Thus, bacteriocins might play a significant role on a microecological scale in dental plaque *in vivo*.

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CHAPTER 3

ANTAGONISTIC SUBSTANCES PRODUCED BY STREPTOCOCCI FROM HUMAN DENTAL PLAQUE AND THEIR SIGNIFICANCE IN PLAQUE ECOLOGY

INTRODUCTION

Streptococci constitute one of the numerically dominant genera in dental plaque. *Streptococcus mutans* will cause caries in monoassociated rats (6,21) and possibly is etiologically associated with human dental caries (26,40).
Many factors influence dental plaque formation, such as selective adherence of strains to tooth surfaces (15,28,29,31), aggregation of cells (13) and dietary supplies (7,22,24,41). The microbial composition of the established dental plaque may be controlled by nutrient requirements (3) or production of antagonistic substances. Some of these substances may be metabolic end products, like peroxide (5,14) or acid (5,8,35). In addition, evidence has been obtained that bacteriocins are widely produced by oral streptococci (11,12,19,20,33,36-39), but up to now no direct evidence for a functional role in plaque ecology has been obtained.
Since reported studies on antagonistic substances from oral streptococci have so far dealt only with selected strains or covered only single species, it was our aim to investigate the possible role of bacteriocins as a regulating factor in intra- as well as interspecies relations of a large number of clinical isolates of streptococci from homologous and heterologous plaque samples.

MATERIAL AND METHODS

Bacterial strains
The strains used were isolated from approximal plaque samples from buccal and lingual sites of the left and right upper molars of patients at the dental clinic of the University of Nijmegen. To study the production and characteristics of antagonistic substances 69 streptococcal strains were isolated from

10 patients and identified as *S. mutans* (15 strains), *S. sanguis* (30 strains) and *S. salivarius* (10 strains) in addition to 14 not identified streptococcal strains. Activity was tested against a variety of indicator strains, obtained from several laboratories and from laboratory stock.
To characterize the relationship between *S. mutans* and *S. sanguis* another 46 *S. mutans* strains (all serotype c, except for two strains belonging to serotype f and g) and 71 *S. sanguis* strains were isolated from defined plaque samples of 15 patients.
Since production of antagonistic substances by a number of strains appeared to decrease by subsequent transfers after isolation, antagonistic activity was tested within two weeks and after not more than two transfers following primary isolation.

Media
Strains were routinely kept on blood agar plates containing brain heart infusion (Oxoid) 2.5%, Bacto-pepton (Difco) 1%, glucose 0.05%, KNO_3 0.1% and agar (Difco) 2.5%, completed with 10% (v/v) sheep blood. Strains were subcultured each fortnight.
Production of antagonists was demonstrated on TSY agar, containing trypton soy broth 3% and yeast extract 1%, on brain heart infusion (BHI) agar or on Todd-Hewitt (TH) agar (all from Oxoid).
To investigate the effect of carbohydrate, TH agar was supplied with 2.5% sucrose or the medium was composed from separate constituents with the exception that sucrose was replaced by 0.4% glucose.
All media were solidified with 1.5% agar (Difco).

Isolation and identification
Small pieces of dental plaque material were removed with a sterile dental scaler, suspended in 5 ml of 0.85% NaCl solution, and dispersed by ultrasonic treatment for 30 sec. with a microtipped Branson sonifier at 75 Watt. Serial dilutions were spread on mitis-salivarius agar (Oxoid). Separate streptococcal

colonies appearing on these plates were selected and identified by testing the ability to ferment mannitol and sorbitol (*S. mutans*) and to hydrolyze arginine (*S. sanguis*) and aesculin (*S. mutans* and *S. salivarius*). Supernatants of 48 hour cultures on thioglycollate broth (Difco) supplemented with 5% sucrose were used for selective precipitation of dextran (*S. mutans* and *S. sanguis*) or levan (*S. salivarius*). To 0.5 ml of supernatant 4.5 ml of 10% Na-acetate was added, followed by 6 ml or 12.5 ml respectively of ethanol (96%). After standing at room temperature for three hours dextran was precipitated at low as well high ethanol concentration, while levan required a high ethanol concentration for precipitation (10).

Demonstration of antagonistic activity

Production of antagonists in agar plates was tested as described previously (16). Stab inoculations of overnight broth cultures into agar plates were incubated for 48 hours at 37°C in B&T jars (Searle Comp.) under 90% N_2 plus 10% CO_2 atmosphere. Routinely four strains were inoculated into the same plate. After this time, overnight broth cultures of the indicator strains, diluted 100-fold into fresh medium were sprayed over the plates, which were incubated for an additional 24 hours. Clear zones in which growth was absent appeared around inoculated spots of producer strains.

Antagonists production in broth cultures was tested by growth of producer strains for 48 hours in several liquid culture media under anaerobic conditions. Cells were subsequently removed by centrifugation for 15 min at 8000 x *g*, and 0.1 ml of the supernatants were pipetted in wells punched in TSY agar plates, seeded with a suitable indicator strain (about 10^5 colony forming units per plate).

Properties of the antagonistic substances

Heat sensitivity of antagonists was tested by placing the plates in an oven at 70°C for 30 minutes, after the initial incubation. After cooling the plates were sprayed with indicator strains and incubated further. Sensitivity to several enzymes was tested by spraying the plates after the initial

TABLE 1. PERCENTAGE OF CLINICALLY ISOLATED ORAL STREPTOCOCCI PRODUCING ANTAGONISTIC SUBSTANCES ON BHI AGAR, AND AVARAGE DIAMETER (WITH STANDARD DEVIATION) OF THE INHIBITION ZONES PRODUCED BY POSITIVE STRAINS [a].

		producer strains			
		Strep. mutans	*Strep. sanguis*	*Strep. salivarius*	unidentified streptococci
No. strains tested					
Indicator strains					
Escherichia coli K12	%	0.0	25.6	10.0	28.6
	mm	-	1.0(0.0)	0.5(0.0)	0.7(0.4)
Staphylococcus aureus NCTC9752	%	13.3	16.7	20.0	57.1
	mm	1.0(0.0)	1.6(0.9)	5.5(1.9)	5.9(5.6)
Streptococcus faecalis ATCC9790	%	13.3	3.3	20.0	0.0
	mm	1.0(0.0)	2.0(0.0)	3.0(0.0)	-
Streptococcus mutans OMZ61	%	6.7	23.3	10.0	50.0
	mm	4.0(0.0)	2.1(1.3)	1.0(0.0)	2.9(5.7)
Streptococcus sanguis Ny101	%	66.6	16.7	50.0	35.7
	mm	3.5(2.6)	2.4(1.3)	0.9(0.3)	5.2(7.3)
Streptococcus salivarius SW55	%	60.0	6.7	30.0	28.6
	mm	2.6(1.7)	3.0(0.0)	6.0(1.2)	5.7(10.0)
Streptococcus mitis S3	%	86.7	10.0	80.0	21.4
	mm	3.0(2.8)	1.5(0.9)	1.8(2.8)	6.0(11.9)
Actinomyces viscosus Ny1	%	100.0	16.7	10.0	35.7
	mm	5.0(1.7)	4.8(1.8)	3.0(0.0)	4.6(2.2)
Bacterionema matruchotii ATCC14266	%	66.6	23.3	0.0	57.1
	mm	1.8(1.6)	7.0(2.1)	-	7.5(10.8)

[a] Strains showing sharply edged inhibition zones of at least 0.5 mm in diameter were considered to be positive.

incubation with filter sterilized solutions of the enzymes (1mg/ml), incubation for 2 hours at 37°C and subsequent spraying with indicator strains.
The following enzymes were used: trypsin (Merck, Darmstadt, 80.000 units/g), pronase (Calbiochem, 120.000 units/g) and lipase (Boehringer, Mannheim, 6000 units/mg).
To assess the diffusible character, 48 hour macrocolonies were covered with autoclaved sheets of dialysis membrane (Union Carbide, Chicago) and overlayered with BHI supplied with 0.8% agar, seeded with 10^6 colony forming units of the indicator strain per ml.
Hydrogen peroxide production was measured by inoculation of the bacteria on MDO agar (42) or heated blood agar plates (27), followed by incubation for 48 hours at 37°C. Peroxide formation results in clearing and green discolorization of zones around the bacterial spots on the respective media.
In order to establish the presence of phages, the infectivity of agar blocks from inhibition zones was tested according to Hamada and Ooshima (12).

Sensitivity of indicator strains to acids
Holes with 9mm diameter were punched in TSY agar plates seeded with 10^6 colony forming units of the strain under investigation. 0.1 ml of two-fold serial dilutions of acetate/acetic acid or lactate/lactic acid, both of pH 4.2 or 6.0 were pipetted into the holes and the plates were incubated for 24 hours at 37°C under N_2/CO_2 atmosphere. Minimal inhibitory concentrations of the agents in the wells were calculated by plotting the total area of inhibition zones against $\log_2$ of the dilution rate and extrapolation to zero inhibition.

RESULTS

Production of antagonistic substances
Preliminary experiments had shown that a relatively high level of antagonism was attained on BHI medium. In Table 1 the percentage of strains of each species producing antagonistic substances and the average diameter of the inhibition zones is

shown. Considerable differences between the various species may be observed: *S. mutans* strains are relatively inactive against genetically as well as ecologically unrelated strains like *Escherichia coli*, *Staphylococcus aureus* and *S. faecalis*, and also against other strains of *S. mutans*.

TABLE 2. DIAMETER OF INHIBITION ZONES (in mm) PRODUCED ON BHI AGAR BY SOME REFERENCE STRAINS AGAINST THE INDICATORS USED.

	producer strains											
	Streptococcus mutans										*S. sanguis*	
Indicator strains	GS5	OMZ61	OMZ176	C67-1	C67-25	BHT	130	GF71	PK1	E49	Ny101	OMZ9
Escherichia coli K12	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus aureus NCTC9752	0	0	0	0	0	7	0	5	0	0	1	0
Streptococcus faecalis ATCC9700	0	0	0	0	0	5	0	4	0	0	0	0
Streptococcus mutans OMZ61	0	0	0	0	0	0	0	0	0	0	0	2
Streptococcus sanguis Ny101	2	0	0	3	2	4	0	9	0	0	0	1
Streptococcus salivarius SW55	0	0	0	0	0	5	0	3	0	0	0	0
Streptococcus mitis S3	2	0	0	8	2	4	0	7	0	0	0	2
Actinomyces viscosus Ny1	6	2	0	2	3	3	0	8	0	0	0	7
Bacterionema matruchotii ATCC14266	15	0	0	0	11	15	4	0	8	0	4	0

Within the same plaque sample a somewhat lower number of antagonistic relations may be observed, when compared with strains from different origins. Also, the average diameter of inhibition zones was significantly smaller with strains isolated from the same oral plaque sample, compared to strains isolated from the same oral cavity but different plaque samples ($P_t<0.005$) or strains isolated from different individuals ($P_t<0.01$). No significant differences were found when strains from different plaque samples and individuals were compared.

TABLE 3. INHIBITION OF GROWTH OF INDICATOR STRAINS BY CONCENTRATIONS OF LACTATE AND ACETATE AT TWO pH-VALUES IN WELL-DIFFUSION TEST.

	minimal inhibitory concentration (%w/v) in well			
	acetate		lactate	
Indicator strains	pH 4.2	pH 6.0	pH 4.2	pH 6.0
Escherichia coli K12	2.3	12	14	>20
Staphylococcus aureus NCTC9752	1.3	2.5	14	>20
Streptococcus faecalis ATCC9790	2.3	22	18	>20
Streptococcus mutans OMZ61	3.3	>40	14	>20
Streptococcus sanguis Ny101	1.0	8	8	18
Streptococcus salivarius SW55	3.0	30	14	>20
Streptococcus mitis S3	1.4	7	8	>20
Actinomyces viscosus Ny1	1.8	14	10	>20
Bacterionema matruchotii ATCC14266	2.0	8	10	>20

TABLE 4. MUTUAL ANTAGONISTIC ACTIVITY OF *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SANGUIS* STRAINS

	Strep. mutans versus *Strep. sanguis*[a]		*Strep. sanguis* versus *Strep. mutans*[a]	
no. of strains tested for activity	46		71	
no. of strains active	44	(96%)	12	(17%)
no. of combinations tested	626		205	
no. of combinations with inhibition zones	520	(83%)	39	(19%)
average diameter (mm) of inhibition zones (± SD)	4.71	(1.74)	3.20	(1.89)

[a] Activity was tested against a range of arbitrarily choosen indicator strains from the same and unrelated plaquesamples

TABLE 5. ANTAGONISTIC ACTIVITY OF *STREPTOCOCCUS MUTANS* AGAINST *STREPTOCOCCUS SANGUIS* STRAINS ISOLATED FROM THE SAME AND DIFFERENT ORIGINS

origin of the strains	no. combinations tested	percentage of combinations with inhibition	average diameter (mm) of inhibition zones (with standard deviation)
a. plaque sample versus same plaque sample	58	81.0	3.97(1.88)
b. plaque sample versus rest of same oral cavity	143	84.5	5.00(2.77)
c. plaque sample versus total samples from other 14 individuals	353	85.0	4.70(2.13)
d. oral cavity versus same oral cavity	205	84.0	4.60(2.39)
e. oral cavity versus total samples from other 14 individuals	376	85.0	4.68(2.11

However, bacteriocin-like substances are often produced against other oral streptococci and against the plaque microorganisms *Actinomyces viscosus* and *Bacterionema matruchotii*. The former appeared to be extremely sensitive to *S. mutans* products. *S. sanguis* rarely produces substances against other microorganisms, including oral streptococci. *S. salivarius* shows intermediate characteristics: the tested oral streptococci, except *S. mutans* are inhibited whereas both actinomysetes are relatively resistant. Within the group of unidentified streptococci, substances are produced in varying amounts against all tested strains except *S. faecalis*, possibly indicating the heterogeneity of this group.

A number of reference strains, obtained from several institutes were tested against the same group of indicator strains. The results are shown in Table 2.

To check whether the inhibition zones might be due to the production of latic and volatic acids, the indicator strains used throughout this study were tested for sensitivity to acetic and latic acid at pH 4.2 and 6.0 (Table 3). Comparison with Table 1 shows that only a partial correlation between sensitivity to acid and antagonistic substances produced by oral streptococci can be observed. This is particulary evident at pH 6.0, a value commonly found in streptococcal inhibition zones.

Interaction between S. mutans and S. sanguis

As was shown before, *S. mutans* produces often antagonistic substances against *S. sanguis*. 96% of the tested strains inhibited at least one of the *S. sanguis* indicator strains, and 83% of the tested combinations resulted in inhibition (Table 4). Of *S. sanguis* strains, only 17% were active against *S. mutans* and 19% of the combinations yielded inhibition zones. Moreover, the sizes of inhibition zones were significantly smaller ($P_t < 0.001$).

To check whether *S. mutans* strains originating from a certain plaque sample or oral cavity would display a different level of antagonism towards *S. sanguis* from the same or different sites, interactions were scored according to these criterions (Table 5).

TABLE 6. MUTUAL INHIBITORY ACTIVITY ON TSY AGAR OF *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SANGUIS* STRAINS ISOLATED FROM THE SAME ORAL CAVITY OR THE SAME PLAQUE-SAMPLE

	indicator strains											
	Strep. sanguis								*Strep. mutans*			
Producer Strains	B2	B6	B7	B13	B14	B15	A5	A7	A3	A4	A8	C1
Strep. sanguis												
B2	-	-	-	-	-	-	0	0	0	0	0	0
B6	0	-	0	2	1	0	0	1	0	0	0	0
B7	0	0	-	0	0	0	0	0	2	0	0	2
B13	0	0	0	-	2	0	0	0	0	0	0	0
B14	0	0	0	0	-	0	0	1	0	0	0	0
B15	0	-	-	-	0	-	0	0	0	0	0	0
A7	0	0	0	1	0	0	0	-	0	0	0	0
Strep. mutans												
A3	4	0	4	4	5	4	4	5	-	0	0	0
A4	4	0	0	2	4	0	2	2	0	-	0	0
A8	4	5	4	5	12	2	3	13	1	0	-	0
C1	4	2	8	5	5	4	2	4	1	0	0	-

Values represent diameter of inhibition zone in mm.

A, B and C refer to different plaquesamples.

The circumlined section of the table represents interactions between strains from the same plaquesample.

- not tested.

TABLE 7. THE EFFECT OF THE CULTURE MEDIUM ON THE PRODUCTION OF ANTAGONISTIC SUBSTANCES ON SOLID MEDIA

	producer strains[a]			
	Strep. mutans	*Strep. sanguis*	*Strep. salivarius*	unidentified streptococci
no. of producer strains tested	12	25	6	10
no. of tests[b]	24	57	14	30
media	no. of tests with inhibition zones			
BHI	24(100%)	57(100%)	14(100%)	30(100%)
TSY	24(100%)	20(35%)	13(93%)	13(43%)
TH-glucose	17(71%)	27(47%)	12(86%)	14(47%)

[a] only strains producing activity on BHI agar were tested.

[b] indicators used in test depend on the activity spectra of the producer strain. Some producers were tested against more than one indicator strain.

The interrelations between *S. mutans* and *S. sanguis* strains isolated from the same plaque sample are shown in Table 6. *S. mutans* strains are active almost exclusively against *S. sanguis* and not against other *S. mutans* strains. *S. sanguis* shows only small antagonistic effects against *S. sanguis* as well as *S. mutans* strains.

Characteristics of the production

Strains producing antagonistic substances on BHI agar were tested also on TSY agar and TH-glucose agar against several indicator strains (Table 7). *S. mutans* and *S. salivarius* generally produce equally well on TSY agar, although the sizes of the inhibition zones may vary. Production, however, was

TABLE 8. ANTAGONISTIC RELATIONS BETWEEN BACTERIA FROM DENTAL PLAQUE IN THE PRESENCE (TH-SUCROSE AGAR) OR ABSENCE (TH-GLUCOSE AGAR) OF SUCROSE

	number of antagonistic relations[a] producer strains				
indicators	*Strep. mutans*	*Strep. sanguis*	*Strep. salivarius*	unidentified streptococci	total
Strep. mutans OMZ61	1(1)	-	0(1)	2(2)	3(4)
Strep. sanguis Ny101	10(11)	3(7)	4(6)	2(3)	19(27)
Strep. salivarius SW55	1(1)	-	1(1)	-	2(2)
Strep. mitis S3	0(1)	-	2(3)	-	2(4)
Bact. matruchotii ATCC14266	6(6)	4(4)	-	4(4)	14(14)
Act. viscosus Ny1	10(11)	2(2)	1(1)	-	13(14)

[a] values in parenthesis represent the number of strains tested and active on TH-glucose agar.

- no active strains available.

TABLE 9. PRODUCTION OF ANTAGONISTIC SUBSTANCES IN TSY BROTH BY ORAL STREPTOCOCCI ACTIVE ON TSY AGAR

	number of producing strains	
Producer strain	TSY-agar	TSY-broth
Strep. mutans	20	2
Strep. sanguis	9	6
Strep. salivarius	2	0
unidentified streptococci	9	5

somewhat diminished on TH-glucose agar. Both TSY agar and TH-glucose agar restricted the production of antagonistic substances by *S. sanguis* and the unidentified streptococci. Replacement of glucose by sucrose in the medium affected the sensitivity of oral streptococci, but not of actinomycetes, to some of the substances produced (Table 8), suggesting a protective mechanism induced by the presence of sucrose in oral streptococci only.

Although antagonistic substances are abundantly produced by *S. mutans* and *S. salivarius* on solid media, in broth cultures activity could only seldom be observed (Table 9). *S. sanguis* and the unidentified streptococci more frequently produced activity in broth cultures. The activity spectra of the substances produced in broth cultures did not significantly differ from those obtained with the corresponding agar cultures.

Properties of the antagonistic substances

No evidence was obtained for the presence of phages in the inhibition zones.

Under anaerobic conditions, used in the tests on the production of antagonistic substances, none of the tested strains produced detectable amounts of peroxide.

Some of the substances produced, were sensitive to proteolytic enzymes (Table 10) and thus may be bacteriocins. Substances produced by *S. mutans* against *S. aureus* appear to be nonproteinaceous, but most substances inhibiting other plaque microorganisms appear to be proteins. The same tendency is found with *S. salivarius* and the unidentified streptococci. Most of the antagonistic substances of *S. sanguis* on the contrary, are resistant to proteolytic enzymes, thus, appear not to be bacteriocins. Some of the antagonistic substances are sensitive to lipolytic enzymes. Most substances are resistant to heat treatments (30 min 70°C), but 3 out of 6 *S. salivarius* products and 4 out of 20 *S. mutans* products appeared to be sensitive. From Table 11 it can be seen that low as well as high molecular weight substances are produced, since some of the substances do not pass dialysis membrane.

The active substance(s) of one producer strain, inhibiting

TABLE 10. SENSITIVITY OF ANTAGONISTIC SUBSTANCES TO TREATMENT WITH PROTEOLYTIC ENZYMES

indicators	number of antagonistic substances sensitive to proteolytic enzymes[a] producer strains				
	Strep. mutans	*Strep. sanguis*	*Strep. salivarius*	unidentified streptococci	total
Staph. aureus NCTC9752	0(7)	0(9)	NT	1(8)	1(24)
Strep. mutans OMZ61	1(2)	NT	1(2)	1(2)	3(6)
Strep. sanguis Ny101	2(3)	0(1)	NT	NT	2(4)
Strep. mitis S3	1(2)	NT	6(6)	NT	7(8)
Bact. matruchotii ATCC14266	2(3)	0(3)	NT	2(4)	4(10)
Act. viscosus Ny1	15(17)	1(1)	NT	NT	16(18)
Total	21(34)	1(14)	7(8)	4(14)	

[a] values in parenthesis represent the number of strains producing antagonistic substances against the indicator strain tested.

[b] NT = not tested

TABLE 11. DIFFUSIBILITY OF ANTAGONISTIC SUBSTANCES THROUGH DIALYSIS MEMBRANE

Producer strain	no. of substances tested	no. of diffusible substances
Strep. mutans	20	12
Strep. sanguis	20	18
Strep. salivarius	6	0
unidentified streptococci	11	9

quite different bacteria, mostly had identical characteristics with respect to sensitivity to proteases, heat and diffusibility. Some strains, however, clearly produced more than one antagonistic substance, with distinguishable physicochemical properties.

DISCUSSION

Ample evidence, as obtained in various studies, has unquestionably proved the frequent production of antagonistic substances among oral streptococci. However, the nature of the inhibiting substances is still a matter of discussion. Bacteriocin-like substances were first reported by Kelstrup and Gibbons (19) and studied more in detail by the same authors (20) and others (11, 12,36,37). Acids and hydrogen peroxide (5,14,25) were also recognized as antagonistic agents of oral streptococci. In our experiments, carried out under anaerobic conditions, hydrogen peroxide appeared to be an improbable antagonistic determinant. A similar conclusion was drawn by Hamada and Ooshima (12). Acid production may have caused inhibition zones in some of our experiments, but is probably not the principal agent. The pH-values within the inhibition zones were mostly around 6.0, a value tolorated by the indicator strains used. Donoghue and Tyler (5) discussed the possible role of undissociated acids as inhibitory agents amongst oral streptococci, as it was shown to be for Enterobacteriaceae (1,43). However, the sensitivity of the indicator strains to the principal acids produced by oral streptococci only partly correlated with sensitivity of the antagonistic substances produced. Strong evidence for bacteriocins as antagonistic agents is obtained from the sensitivity of a considerable proportion of the products of *S. mutans*, *S. salivarius* and the unidentified streptococci to proteolytic and sometimes in addition to lipolytic, enzymes. On the contrary, *S. sanguis* products generally appeared to be resistant, which confirms their different nature.
Hamada and Ooshima (12) noted that reference strains *S. mutans* serotype d did not produce mutacins, while clinical isolates did so. In our experiments fresh clinical isolates producing

antagonistic substances sometimes lost this property after transfers on laboratory media. This might be explained by a spontaneous loss of the genetic determinant (possibly a plasmid) for bacteriocin-production, as was shown to occur in gram-negative microorganisms (34) and staphylococci (17).
Two strains of *S. mutans* produced antagonistic substances also in broth cultures. Recently this was also found by others (4, 12,32), however, under special nutritional conditions. Schlegel and Slade (38) obtained activity from broth cultures of *S. sanguis* (strain Challis). *S. mutans* SW31, a clinical isolate, was shown to produce a mutacin of lipoprotein nature with bactericidal effects on almost exclusively *S. sanguis* strains. Probably it exerts an effect on the cellular membrane by inducing changes in permeability and hence inhibition of most energy-dependent processes (Weerkamp, unpublished data). It thus resembles bacteriocins from other gram-positive organisms (17), including *S. sanguis* (38). Some *S. sanguis* strains produced activity in broth cultures against *S. aureus*, but not against streptococci.
Although it was shown that bacteriocins are produced *in vitro*, the question remains to be answered if they play a major role in regulating dental plaque flora *in vivo*. Rogers (37) showed that streptococci able to produce extra-cellular polysaccharide (EPS), are protected against the action of bacteriocin-like substances on sucrose containing media. In a few cases a substantially decreased sensitivity of streptococci could indeed be observed. In the present study EPS-producing *A. viscosus* was not protected. Also, it has been shown that bacteriocin-based antagonism between oral streptococci can regulate the microbial composition of *in vitro* plaque deposits (Chapter 2). Although they are sensitive to several proteolytic enzymes, mutacins were not destroyed by plaque proteases, according to Hamada and Ooshima (12). The number of bacteriocins or the amount of bacteriocin produced appear to be affected markedly by nutritional conditions, as was also found by Rogers (36). However, as bacteriocins will act primarily on a micro-ecological scale and, as a few molecules are sufficient to kill sensitive strains, relatively low titers are probably sufficiént to express their

full action in dental plaque.

The slightly, but significantly, lowered level of antagonism found with strains isolated from the same plaque sample, compared with strains from different sites, may result from a shift in the composition of the population due to the action of the antagonistic substances.

Incidence, inhibition spectra and physicochemical properties of the antagonistic substances produced by different streptococcal species from the same habitat, appeared to be characteristic to these species. This implies that these, partly bacteriocin-like, substances may effectively influence the interspecies relations of plaque microorganisms and may favourably contribute to the persistence of distinct species in plaque.

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CHAPTER 4

PRODUCTION, PURIFICATION AND SOME PROPERTIES OF STAPHYLOCOCCIN 1580, A BACTERIOCIN OF *STAPHYLOCOCCUS EPIDERMIDIS*

INTRODUCTION

Bacteriocins are proteinaceous bactericidal substances produced by bacteria. The production of bacteriocins is a widespread phenomenon among probably all bacterial species (22,23). Some of them, especially the colicins, have been purified and their mode of action has been studied into detail. Bacteriocins of gram-positive bacteria have also attained much attention (for review, see 23) but are generally less thoroughly investigated. Staphylococcins were first described by Fredericq (4) and distinguished into a number of different types on basis of their inhibition spectrum. Similar classifications have been made by other investigators (8,12,21). Some of the staphylococcins were purified and characterized (5,6,9,10) and their mode of action was investigated (3,7,11,13,14).

A staphylococcin produced by *Staphylococcus epidermidis* 1580 was studied in this department, and purified to homogeneity (9). It was produced on a complex semisolid medium and characterized as a complex molecule, consisting of protein (42%), carbohydrate (34%) and lipid (22%) (10). In addition, various physicochemical properties were investigated.

We now describe a production and purification procedure based on a semisynthetic liquid medium and some additional properties of the bacteriocin.

MATERIALS AND METHODS

Bacterial strains and media

The staphylococcin 1580-producing strain was originally obtained from T. Lachowicz, Krakow, Poland. The strain is coagulase negative, does not ferment mannitol, and is not hemolytic. *S. aureus* Oxford 209P was used as the indicator strain. Both strains were grown on tryptone soya agar (Oxoid) and subcultured each fortnight. Medium A, containing 1% casein hydrolysate (enzymatically

hydrolyzed, NBC) , 20 mM potassium phosphate (pH 7.0), 1 mM magnesium sulfate, 2 mM cysteine, 2 mM histidine, 20 µM thiamine and 1 ml of the oligodynamic solution described by Pollock and Kramer (20) per liter, was used as the basic semisynthetic medium. The medium was supplemented with various carbon sources in 1% final concentration, unless otherwise indicated.

Bacteriocin assay

The staphylococcin activity was determined either as described previously (9), or by using the method described here. 0.1 ml amounts of serial twofold dilutions of a solution containing bacteriocin activity were pipetted into wells (9 mm diameter) cut into tryptone soya agar plates that were previously seeded with the indicator strain (about 10^7 cells/plate). After overnight incubation the approximate surface area of the resulting inhibition zone, measured as the square of its radius, subtracted by 20 (=square of the radius of the well), was plotted against $\log_2$ of the dilution factor (Fig. 1). The reciprocal of

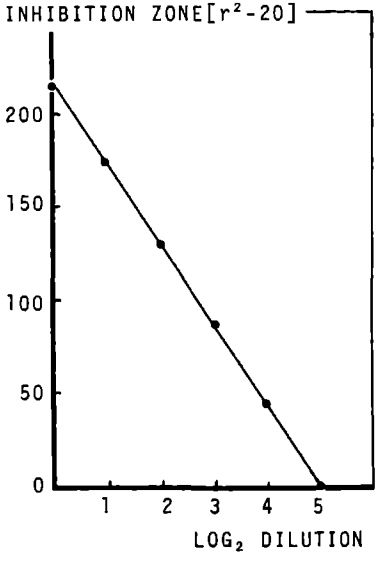

Fig. 1. Quantitative determination of the staphylococcin 1580 activity. Serial dilutions (0.1 ml) were pipetted into wells (9 mm diameter) punched in TSA plates, previously seeded with 10^7 colony forming units of *S. aureus* Oxford 209P. After overnight incubation the surface area of the inhibition zones was measured, and plotted against the dilution factor. The reciprocal of the dilution at the dissection point with the dilution axis was taken to be the activity in arbitrary units per 0.1 ml.

the apparent dilution at the dissection point of the resulting straight line with the dilution axis, was taken as the bacteriocin titer. The activity was expressed as arbitrary units (A.U.) per ml. Very similar results were obtained with both methods. The latter method was preferred because of its convenience.

Column isoelectrofocussing

Isoelectrofocussing in a sorbitol gradient was performed with a LKB 8100-1 Ampholine column with carrier ampholytes of pH 6-11, operated at 12 Watt for 48 h. Fractions (about 2.6 ml) were collected and assayed for staphylococcin activity, protein (absorbance at 280 nm) and pH.

Protein determination

Protein was measured according to the method of Lowry (19) with bovine serum albumin as standard.

RESULTS

Production of staphylococcin 1580

It was previously shown that optimal production of staphylococcin 1580 was achieved on semisolid trypticase soy medium, and no activity was found in a simple synthetic medium (9). However, we observed a striking effect of the carbon source applied to a semisynthetic basal medium on the production of staphylococcin 1580 (Table 1). No correlation was found between the cell yield obtained with various carbon sources and the staphylococcin 1580 production, but a direct relationship between the staphylococcin concentration in the medium and the final pH of the medium is obvious (Fig.2). Moreover, neutralizing the pH of a stationary, glucose-grown culture markedly increased the amount of bacteriocin found in the culture fluid within 1 h (Table 1). To investigate the possibility that the pH might affect the amount of bacteriocin adsorbed by the cells, the cell pellets were extracted with 6 M urea and the bacteriocin concentration in the extracts was determined (Table 1). The activity in extracts of the pH-adjusted culture

TABLE 1. EFFECT OF THE CARBON SOURCE IN THE MEDIUM ON THE PRODUCTION OF STAPHYLOCOCCIN 1580

Carbon source	Yield (mg dry wt cells/ml)	Final pH	Staphylococcin 1580 concentration (arbitrary units/ml)	
			medium	urea extract
none	0.34	6.60	52	-
mannitol	0.25	6.59	67	-
sorbitol	0.51	6.53	52	-
glycerol	0.80	5.07	24	-
acetate	0.37	6.70	100	-
glycerol+ acetate	1.00	5.56	24	84
pyruvate	1.24	7.36	224	130
glucose	0.84	4.87	14	10
glucose (pH adjusted)[a]	0.82	6.89	43	94

S. epidermidis 1580 was grown overnight at 37°C with vigorous aeration in medium A supplemented with the carbon sources given above (1% final concentration). The cells were harvested by centrifugation and resuspended 10 times concentrated in 0.2M potassium phosphate (pH 7.0) and 6M urea, and kept at 0°C for 30 min. Then the cells were centrifuged and discarded. The staphylococcin concentration in the culture supernatant and the urea extract was determined.

[a] after overnight growth the pH of the culture was adjusted to about 7, and the culture was further incubated at 37°C for 1 h, after which it was treated as described.

- not tested

appears to be increased over that found in the culture that had not been neutralized. Similar results as described here were obtained by extraction with 5% NaCl. Moreover, a pH-range from 5 to 8 did not affect the adsorption of staphylococcin 1580 to cellulose powder (results not shown). Therefore, the production of staphylococcin 1580 appears to be depressed at low external pH.

Activity of staphylococcin 1580 in the culture medium is detec-

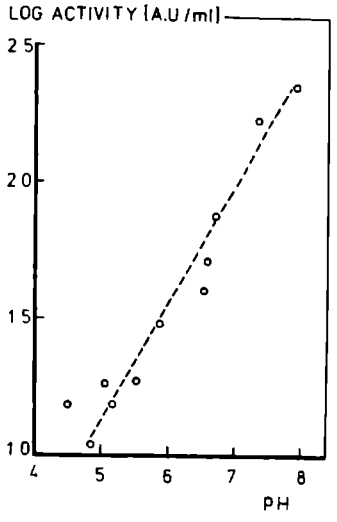

Fig. 2. Effect of the final pH of the medium on the production of staphylococcin 1580. *S. epidermidis* 1580 cells were grown on various media as described in Table 1. The final pH of the culture supernatants are plotted against the staphylococcin 1580 titer.

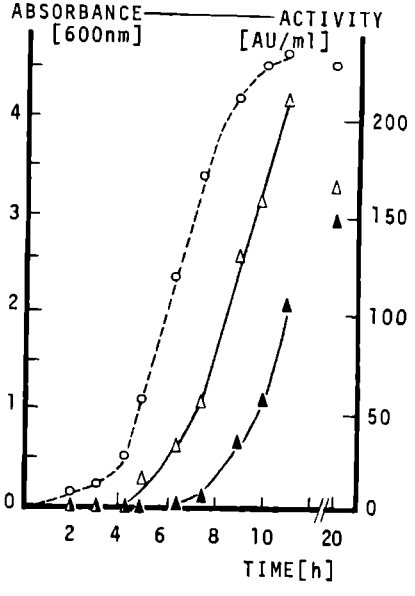

Fig. 3. Effect of the growth phase on the production of staphylococcin 1580. *S. epidermidis* 1580 cells were grown in medium A with 1% sodium pyruvate, at 37°C with vigorous aeration. At various intervals samples were removed, and treated as described in Table 1.
Symbols: o, growth, absorbance at 600nm;
Δ, staphylococcin 1580 concentration in culture supernatant;
▲, staphylococcin 1580 concentration in 6M urea cell extract (10x concentrated).

ted first at the end of the exponential phase of growth, and inclines rapidly until growth ceases (Fig. 3). Activity in 6M urea extracts of cell pellets is only observed at later stages of growth. Moreover, upon prolonged incubation up to 24h, the activity in the medium decreased, while that associated with the cells further increased. This suggests that the cell-associated staphylococcin activity, extractable with 6M urea, results from secondary adsorption of the bacteriocin to the cells.

Purification of staphylococcin 1580

S. epidermidis 1580 was grown at 37°C for 18h in medium A with 1% sodium pyruvate as carbon source in a New Brunswick gyratory shaker. All subsequent steps were carried out at 4°C. The scheme shown in Fig. 4 was followed, except that the Sephadex G50-gel filtration step occasionally was omitted, since the activity co-eluted with the single protein peak observed.

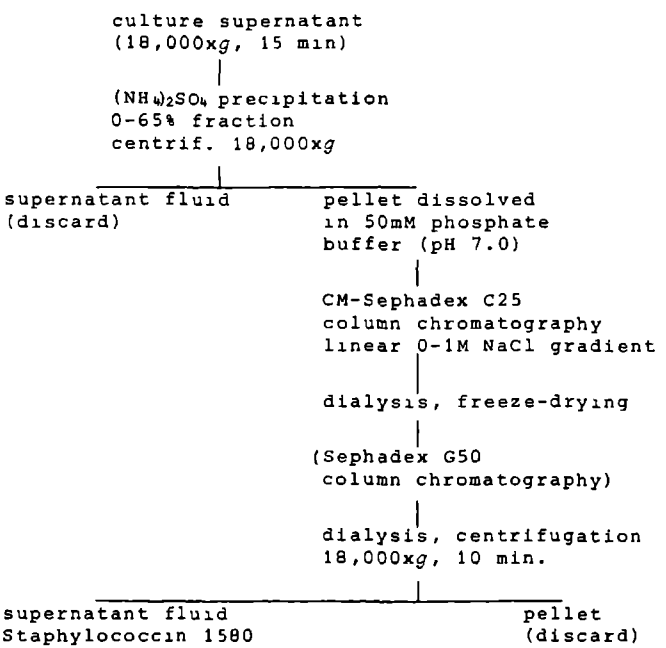

Fig. 4. Purification scheme of staphylococcin 1580.

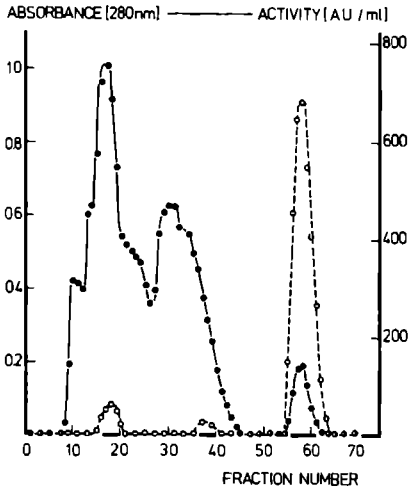

Fig. 5. CM-Sephadex C25 ion exchange chromatography of staphylococcin 1580. The elution occurred with a linear 0-1M NaCl gradient in 50 mM potassium phosphate buffer (pH 7.0). Each 7 ml fraction was assayed for staphylococcin activity (o) and protein (•). Fractions 56 through 64 were pooled, yielding 60% recovery of the total activity applied to the column.

1.5 l of culture supernatant was brought to 65% saturation by slowly adding powdered ammonium sulfate under constant stirring. After about 30 min the precipitate was collected by centrifugation, dissolved into 120 ml of 50mM potassium phosphate (pH 7.0) and applied to a CM-Sephadex C25 column (2.5x40 cm) prepared in the same buffer (Fig. 5). The column was eluted with 600 ml of a linear 0-1M NaCl gradient in phosphate buffer. Staphylococcin 1580 bound strongly to the column and was eluted at about 0.5M NaCl. Small amounts of activity were observed with the major protein peaks and probably represent bacteriocin aspecifically bound to these proteins. The fractions with a high specific activity were pooled, dialyzed overnight against phosphate buffer (pH 7.0) followed by another 6h against distilled water, and lyophillized. The freeze-dried preparation was dissolved in 10 ml of buffer and either applied to a Sephadex G50 column (2.0x 40 cm)(not shown) or only centrifuged for 10 min at 18,000 x *g* and again dialyzed. A small precipitate,containing no bacteriocin activity was discarded. The final preparation was homogeneous on SDS-polyacrylamide gel electrophoresis. The results of this purification are summarized in Table 2.

TABLE 2. PURIFICATION OF STAPHYLOCOCCIN 1580

Purification step	Volume (ml)	Arbitrary units	Protein (mg)	Specific activity (AU/mg)	Recovery (%)	Times purified
culture supernatant	1,5000	250,000	4,200	59	100	1
$(NH_4)_2 SO_4$ precipitation	120	205,000	44	4,540	82	77
CM-Sephadex chromatogr.	60	120,000	3.7	27,000	48	457
dialysis, centrifug.	10	95,000	2.6	36,500	38	745

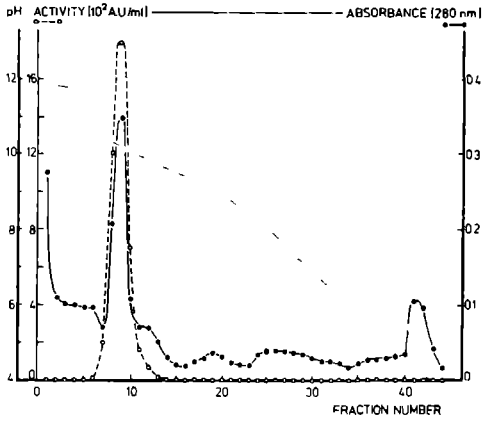

Fig. 6. Column isoelectrofocussing of staphylococcin 1580. Focussing was carried out for 48 h in a sorbitol-ampholine gradient. Each 2.6 ml fraction was assayed for protein (•), staphylococcin activity (o) and pH (dotted line).

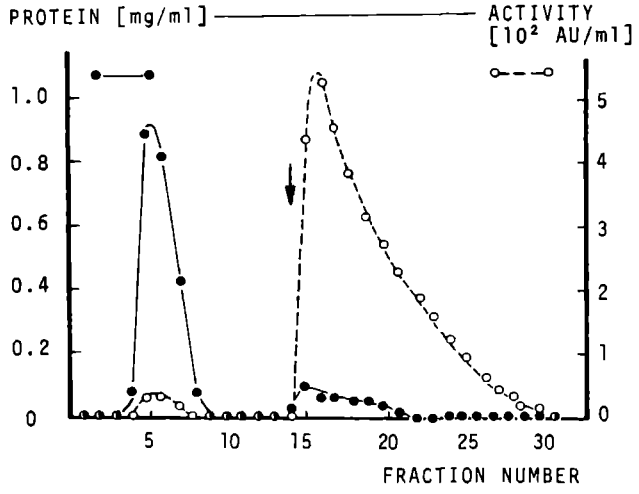

Fig. 7. Cellulose column chromatography of staphylococcin 1580. The elution occurred discontinuously with 50mM phosphate buffer (pH 7.0), followed by 0.2M buffer containing 6M urea. The arrow indicates the elution of the urea front. Each 3 ml fraction was assayed for staphylococcin activity (o) and protein (•). The recovery was 90%.

Isoelectrofocussing

Fig. 6 shows the results obtained upon electrofocussing a staphylococcin 1580 preparation obtained after CM-Sephadex chromatography. The apparent isoelectric point was estimated to be 10.2.

Cellulose column chromatography

Staphylococcin 1580 has a strong and unspecific affinity for cell walls and membranes (10) and various other compounds (not shown). To demonstrate the surface reactive nature of the bacteriocin a staphylococcin preparation obtained after ammonium sulfate precipitation, containing about 9000 AU, was applied to a cellulose column (Whatman standard grade; 1.4x10 cm) prepared in 50mM potassium phosphate buffer (pH 7.0), and eluted at room temperature with 35 ml of the same buffer (Fig. 7). The major part of the proteins applied to the column was eluted, but almost no activity was found. Then the column was eluted with 40 ml of 6M urea in 0.2M phosphate buffer, which resulted in the elution of the bacteriocin activity.

Many bacteriocin-like substances are detected only in solid media, or are produced in liquid media only under highly specialized conditions (see 23). In contrast to our previously reported optimal production of staphylococcin 1580 on a semisolid medium (9), a very good yield of bacteriocin activity was now obtained in a liquid medium containing an appropriate carbon source. This discrepancy can partly be attributed to a depression of the bacteriocin production at low external pH values, which also occurred on the previously used medium. This effect was not the result of different adsorption properties of staphylococcin 1580 at various pH values. The inhibition spectra of the bacteriocins produced by either method are identical. Moreover, mutant strains selected for resistance to one of the preparations are simultaneously resistant to the other.
It is well known that production of bacteriocins may be strongly affected by the composition of the medium, which optimal composition has to be found empirically (23). The production of staphylococcin A was dependent on the amino acid composition of the medium (17) and depressed in the presence of glucose, which was attributed to the resulting low external pH (18). Production of staphylococcin 462 was enhanced by the addition of 0.5% mannitol to a complex medium, whereas that of staphylococcin 414 was decreased (6). The production of phage type 71 staphylococcin was enhanced by either glucose and mannitol (1).
Maximal yield of staphylococcin 1580 was observed in the late exponential and early stationary phase of growth, similarly to that observed for staphylococcin C55 (2) and streptococcin A-FF22 (23). In agreement with our results, various studies reported a substantial loss of staphylococcin activity upon prolonged incubation of cultures (2,16). We showed that this effect may be attributed at least partly to readsorption of the bacteriocin to the producer cells. Several staphylococcins have been found firmly associated with cell components, and are found in the culture fluid both as large complexes and as low molecular weight forms (5,6). In its native state at least a portion of staphylococcin 1580 could also diffuse through a dialysis mem-

brane which was inserted in the agar underneath the producing strain (unpublished observation), whereas the isolated bacteriocin could be dialized without substantial loss of activity. The spontaneous formation of aggregates of purified staphylococcin 1580 was reported previously (9).

Staphylococcin 1580 is a basic protein, a property shared by some of the colicins that act in a similar way, such as colicin E1 (24) and Ia and Ib (15). Lack of data on bacteriocins of gram-positive bacteria prevents as yet a conclusion on a direct relationship between the basic character and the mode of action.

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CHAPTER 5

CONDITIONAL KILLING EFFECT OF STAPHYLOCOCCIN 1580 AND REPAIR OF SUBLETHAL INJURY IN *STAPHYLOCOCCUS AUREUS*

INTRODUCTION

Staphylococcin 1580 is a bacteriocin produced by *Staphylococcus epidermidis* (16,17). In sensitive cells it rapidly affects the permeability of the cell membrane, which results in a simultaneous inhibition of several energy-dependent processes, such as active transport and macromolecular synthesis and in a rapid decline of cellular ATP level (18). Like colicin E1 and K (5,6) and A (19) it exerts its action probably directly on the cell membrane, since amino acid transport in membrane vesicles is inhibited too (19). Staphylococcin 1580 also resembles a number of bacteriocins produced by a variety of other Gram-positive bacteria, such as other staphylococci (3), clostridia (1,15), streptococci (22,30) and lactobacilli (32).

Though numerous studies have been focused on the cellular events after treatment with bacteriocins of this type, the factor(s) causing death, measured by inability to form colonies, have as yet not been fully cleared. Mutant cells lacking the Ca^{2+}, Mg^{2+}-activated ATPase activity (unc A) were killed by colicin K in spite of the absence of an effect on the ATP level and on macromolecular synthesis (26). Recently, Kopecky *et al* (21) showed that colicin E1-or K-treated cells may be rescued by plating on media with appropriate concentrations of potassium and magnesium ions. They concluded that killing results from the failure to restore the specific intracellular concentrations of ions required for proper metabolic functions.

In this chapter the conditions required for an effective killing by staphylococcin 1580 and the repair of sublethal injury are described.

MATERIALS AND METHODS

Bacterial strains

Staphylococcin 1580-producing *Staphylococcus epidermidis* 1580, colicin A-producing *Citrobacter freundii* C31 and the indicator strains *S. aureus* Oxford 209P and *Escherichia coli* K12, were described previously (19). *S. aureus* C55, which produces staphylococcin C55 was kindly donated by Dr. L.M. Wannamaker; the indicator strain used for this bacteriocin was *S. aureus* 502A.

The strains were subcultured each fortnight on tryptone soya agar (Oxoid) and stored at 4°C.

Media

Medium CY (low salt medium) consisted of 1% casein hydrolysate (enzymatically hydrolyzed; NBC), 0.2% yeast extract L21 (Oxoid) and 1.5% agar (Difco). (final pH 6.5) This medium contained less than 2×10^{-3}M of sodium chloride.

The basal semisynthetic medium A was derived from the previously described AJ-1 medium except that casein hydrolysate (enzymatically hydrolyzed) was obtained from NBC and glucose was substituted by various carbohydrates in 1% concentration (18).

In the recovery experiments the medium proposed by Iandolo and Ordal (14) was used, which consisted of 0.25% glucose or sodium pyruvate; 30 mM potassium phosphate buffer (pH 7.2); 0.4 mM L-alanine, L-valine, L-leucine, L-glycine, L-proline, L-hydroxyproline, L-aspartic acid, and L-glutamic acid; 0.15 mM L-methionine, L-phenylalanine, L-tyrosine, L-arginine hydrochloride, L-histidine hydrochloride, and L-lysine hydrochloride.

Production and purification of bacteriocins

Colicin A-C31 was obtained by the modified method of Dandeu (4) as previously described (19). Staphylococcin C55 was produced by the method of Dajani and Wannamaker (2).

Staphylococcin 1580 was produced and purified in a way different from the previously used method (16). *S. epidermidis* 1580 was grown overnight at 37°C under vigorous shaking in 1 l batches

in semisynthetic A-pyruvate medium. The cells were removed by centrifugation for 15 min at 8,000 x *g*. All subsequent treatments were carried out at 4°C. The supernatant was brought to 65% saturation by slowly adding powdered ammonium sulfate under constant stirring. After a further 30 min the precipitate was pelleted by centrifugation for 30 min at 12,000 x *g* and dissolved in about 50 ml of 0.02 M potassium phosphate buffer (pH 7.0). To remove undissolved material this solution was centrifuged for 10 min at 6,000 x *g* and the pellet was discarded. The supernatant was layered on top of a CM-Sephadex C-25 (Pharmacia, Uppsala) column (50 x 1.5 cm) and eluted with a linear 0 - 0.8 M sodium chloride gradient in 0.02 M potassium phosphate buffer (pH 7.0). Fractions (10ml) were collected and assayed for staphylococcin 1580 activity and the absorption at 280nm was monitored for detection of protein. The single peak eluted at about 0.6 M NaCl contained over 80% of the total staphylococcin activity and was pooled, dialyzed overnight against excess 0.02 M phosphate buffer, followed by another 4 h with two charges of deionized water. The dialyzed preparation was freeze-dried and stored at 4°C.

Assay of bacteriocin activity

The bacteriocins were assayed as described previously (16). The activity was expressed in arbitrary units (AU) per ml.

Viable count

Total viable counts after treatment with bacteriocin were determined by rapidly diluting samples 1000-fold into ice-cold tryptone soya broth (TSB), and subsequently plating suitably diluted amounts on agar plates, as indicated in the relevant experiments. The R_t-value was defined as the ratio between the viable counts of treated and untreated bacteria.

Conditions of bacteriocin-treatment

Cells in mid-logarithmic phase of growth were centrifuged at 4°C, washed and resuspended in incubation buffer, which contained 0.05 M potassium phosphate (pH 7.0), 0.05 M sodium chloride and 2×10^{-3} M magnesium sulfate, and stored on ice for

no longer than 2 h. Cell suspensions (1 ml) were preincubated for 5 min at 37°C, subsequently supplied with bacteriocins (10 µl volume) and further incubated for 5 min. After that, samples were rapidly diluted and plated as described.

Recovery

The time course of recovery was followed by diluting staphylococcin 1580-treated cells 1000-fold into the recovery medium under investigation, and further incubation at 37°C in static culture, unless otherwise indicated. At the times indicated samples were withdrawn and plated as described.

Transport of glutamic acid

The assay system was the same as used for staphylococcin 1580-treatment, with the exception that after 5 min preincubation at 37°C [^{14}C]-L-glutamic acid (final concentration 10^{-5}M) was added. Samples (0.1 ml) were withdrawn, rapidly filtered on Millipore filter (0.45 µ) and washed with 4 ml of incubation-buffer. After drying, filters were counted in a liquid scintillation counter.

Chemicals

L-[U-^{14}C]-glutamic acid (270 mCi/mmol) was obtained from Radiochemical Centre, Amersham, Bucks; actinomycin D and mitomycin C from Sigma, St. Louis, Miss.; and 2,4-dinitrophenol from Baker, Deventer, The Netherlands. All other chemicals were reagent grade.

RESULTS

Influence of salts

The viability of sensitive cells, treated with relatively low amounts of staphylococcin 1580 or colicin A, both of which are assumed to act in a similar way (19), was much more reduced when the cells were plated on media containing increasing amounts of sodium chloride, than on low-salt medium (Fig. 1). In particular staphylococcin 1580-treated cells were nearly completely viable when plated on low-salt medium. In contrast, the sodium

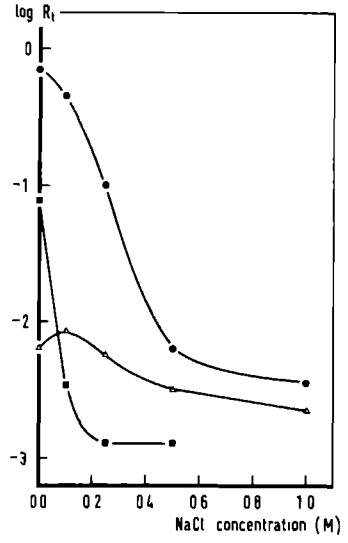

Fig. 1. Effect of sodium chloride on the survival ratio of cells treated with staphylococcin 1580, staphylococcin C55 and colicin A. Mid-logarithmic phase cells of the indicator strain grown on tryptone soya broth (TSB) were centrifuged, washed and resuspended in incubation buffer ($5x10^8$ cells/ml). After 5 min incubation with 100 AU/ml of the bacteriocins the viable count on CY-agar plates (pH 6.5) with various NaCl-concentrations was determined.
●, effect of staphylococcin 1580 on *S. aureus* Oxford 209P;
Δ, effect of staphylococcin C55 on *S. aureus* 502A;
■, effect of colicin A on *E. coli* K12 (V2005).

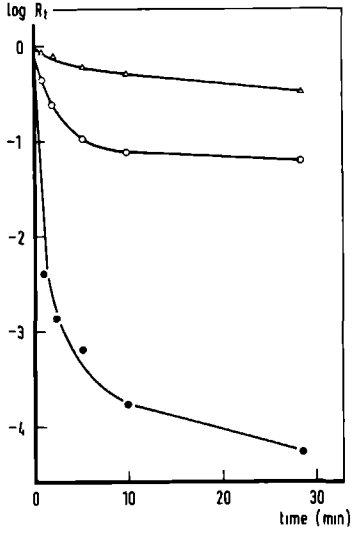

Fig. 2. Effect of the incubation time with staphylococcin 1580 on the survival ratio measured at various NaCl concentrations. Cells of *S. aureus* Oxford 209P, exponentially growing on TSB were centrifuged, washed and resuspended in incubation buffer (pH 7.0) and subsequently treated with staphylococcin 1580 (100 AU/ml) for the indicated time intervals, after which the viable count was determined on Cy-agar plates containing less than $2x10^{-3}$M (Δ), 0.1 M (○) or 1.0 M NaCl (●).

chloride concentration only slightly influenced the survival ratio after treatment with staphylococcin C55.
The effect of salts on the viability after treatment with bacteriocin was investigated more in detail with staphylococcin 1580. Fig. 2 shows that prolonged incubation times hardly further enhance the amount of cells killed at each salt concentration applied.

A variety of compounds had no effect on the survival ratio when present during the staphylococcin-treatment only. Incorporation of salts in the plate medium, however, results in killing ratios characteristic to the salt applied (Table 1). Anions and compounds like leucine and sucrose exert special effect neither on the interaction with the bacteriocin nor on the recovery after injury. Arranging of the cations according to their ion radii strongly suggests a correlation between the radius and the effect in the plate medium in such a way that the more cells are killed the smaller the radius is. The ammonium ion appears to be more lethal than would be expected from this hypothesis, possibly due to the high permeability of its unprotonated form, or to effects on the pH which will be discussed below. Potassium ions, which itself were nearly inert in this system, partly rescued the cells from killing by relatively low concentration of sodium chloride (Fig. 3). Possibly potassium ions compete with sodium ions for the same target or, alternatively their presence may allow the maintenance of a high intracellular potassium concentration required for normal cell function. It should be noted that under conditions of sodium chloride stress, staphylococcal cells accumulate relatively high concentrations of sodium chloride, without distinct effects on the major metabolic functions (31). It is evident from the data presented that increasing concentrations of both cations killed cells progressively. To test whether the used compounds affect certain functions of the cytoplasmic membrane, their influence on glutamate uptake, which was previously shown to be energetically linked to a high energy state of the membrane in *S. aureus* (19), was also investigated (Table 1). Although most salts increased the transport rate significantly over that of

TABLE 1. EFFECT OF VARIOUS SALTS ON THE STAPHYLOCOCCIN 1580 ACTION, THE RECOVERY OF THE TREATED CELLS AND ON GLUTAMATE UPTAKE

Compound added (0.25 M)	Effect on viability (log R_t): salt present during staphylococcin treatment[a] — Plated on CY-agar	Plated on CY-agar+1M NaCl	Salt present in plate medium only[b]	glutamate uptake (nmoles/mg dry weight /min)[c]
–	-0.2	-2.8	-0.2	1.10
$MgCl_2$	-0.2	-1.9	-3.1	2.16
LiCl	-0.3	-2.2	-2.8	1.80
NaCl	-0.2	-2.1	-2.1	2.42
$CaCl_2$	NT	NT	-0.7	NT
KCl	-0.2	-2.6	-0.5	1.48
NH_4Cl	-0.2	-2.2	-1.9	1.54
RbCl	-0.6	-2.9	-0.2	1.18
KCl	-0.2	-2.6	-0.6	1.48
KBr	-0.2	-2.0	-0.5	1.68
KNO_3	-0.2	-2.1	-0.6	1.80
K_2SO_4	-0.2	-2.5	-0.5	1.08
K-acetase	NT	NT	-0.6	NT
Leucine	NT	NT	-0.3	NT
sucrose	-0.2	-2.7	0.2	1.21

[a] The basal incubation medium contained 0.05 M potassium phosphate (pH 7.0), 0.05 M NaCl and $2x10^{-3}$M $MgSO_4$ to which the indicated salts were added.
Washed cells of *S. aureus* 209P ($6x10^8$ cells/ml) were preincubated for 5 min at 37°C after which staphylococcin 1580 was added (100 AU/ml) and incubation continued for 5 min.
[b] Subsequently samples were rapidly diluted more than 1000-fold and plated on the media indicated.
Viable counts after staphylococcin-treatment in basal medium were determined on CY-agar with incorporated salts.
Viable counts aee expressed as the logarithm of the survival ratio (R_t).
[c] Uptake after 1 min by cells suspended in basal incubation medium with the indicated salts was measured and expressed as nanomol per mg dry weight of cells.
NT = not tested.

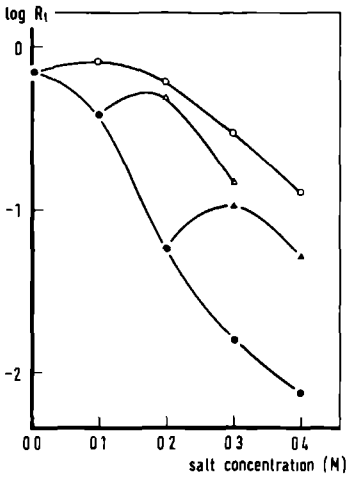

Fig. 3. Effects of mixtures of sodium chloride and potassium chloride on the killing effect of staphylococcin 1580. Conditions were identical to those in Fig. 1. Cells were plated on CY-agar (pH 6.5) with various amounts of NaCl (●) or KCl (○) or mixtures of 0.1 M NaCl plus additional KCl (△) and 0.2 M NaCl plus additional KCl (▲). The indicated salt concentrations refer to final sum of concentrations of both salts.

the basal incubation medium, no correlation was found with their influence on the staphylococcin 1580 killing effect.

Influence of pH

Cells treated with staphylococcin 1580 were plated on low-salt media of various pH values (Fig. 4A). At neutral pH killing was very low under the conditions applied. Towards more acid pH values the killing increased slightly. pH values above 7, however, drastically decreased the survival ratio. This was shown to be an effect on the viability of treated cells, rather than on the interaction of staphylococcin 1580 with the cells, since the latter has a broad range with an optimum at pH 6.5 (Fig. 4B).

Simultaneous influence of pH and salt

Cells treated with staphylococcin 1580 and subsequently plated at neutral pH are sensitive to sodium chloride (Fig. 1 and 5). At slightly alkaline pH this sensitivity of treated cells is significantly increased, whereas at pH 5.5 the survival ratio was high and almost independent of the salt concentration of

the plating medium. These results point out that the effects of salts and pH are interdependent.

Mechanism of recovery

Loss of salt tolerance on staphylococcin 1580-treatment appears concomitantly with a collapse of energy-dependent functions of the cell (18,19). Since treated cells are able to resume growth under favourable conditions, injuries must be repairable. It is shown in Fig. 6 that after a 60 min incubation time treated cells start to recover from the injury, which process is completed within 120 min under optimal conditions. Table 2 gives the results obtained under various incubation conditions and

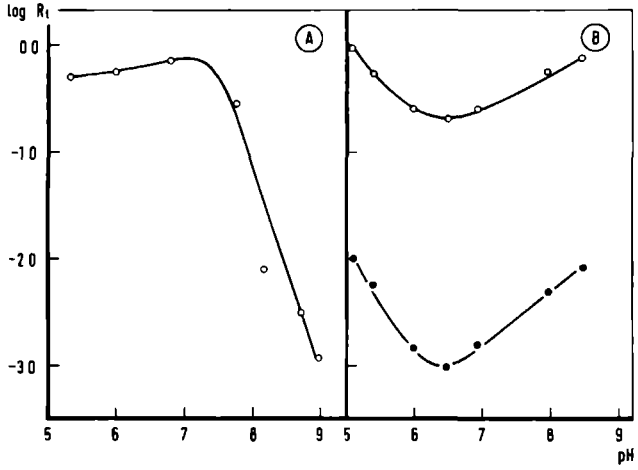

Fig. 4. Effect of pH on the staphylococcin 1580-induced killing
A; washed cells of *S. aureus* Oxford 209P ($5x10^8$ cells/ml) were incubated with staphylococcin 1580 (100 AU/ml) for 5 min and subsequently plated on CY-agar of which the pH was adjusted to the indicated values by the addition of potassium phosphate buffers to a final concentration of 0.05 M. The extreme pH values required the addition of small amounts of HCl or KOH. The potassium ion concentration of the medium however never exceeded 0.1 M.
B; cells were suspended in incubation buffers of various pH ($5x10^8$ cells/ml).
After incubation with staphylococcin 1580 (100 AU/ml) the viable count was determined by plating on CY-agar (pH 6.5) containing either 0.1 M (○) or 1.0 M (●) of sodium chloride.

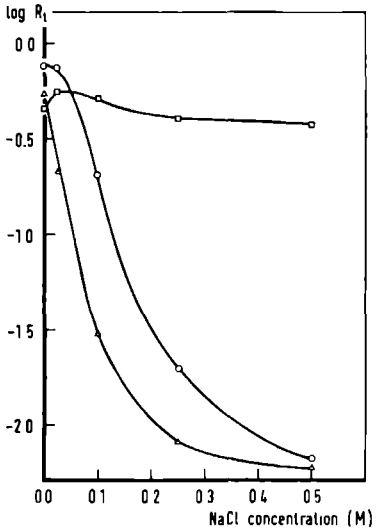

Fig. 5. Influence of the combination of salt and various pH values on the killing effect of staphylococcin 1580. Washed cell suspension of *S. aureus* Oxford 209P (4×10^8 cells/ml) were prepared as described in Fig. 2, and treated with staphylococcin 1580 (100 AU/ml) for 5 min. Viable counts were determined by plating on CY-agar of various pH and NaCl concentration.
The viability of cells not treated with the bacteriocin, was not affected by any of the conditions applied.
□, pH 5.5;
○, pH 6.9;
△, pH 7.6.

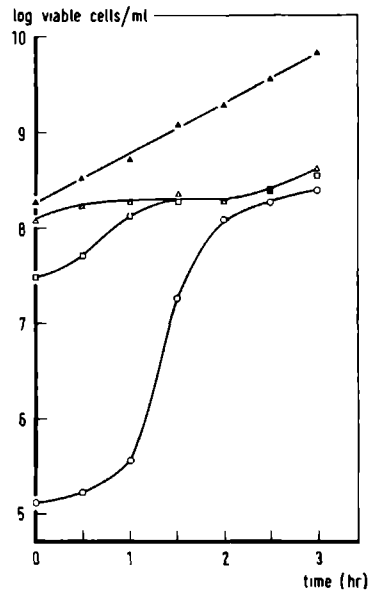

Fig. 6. Recovery of staphylococcin 1580-induced injury. Washed cell suspension of *S. aureus* Oxford 209P (2×10^8 cells/ml) were prepared as described in Fig. 2 and treated with staphylococcin 1580 (100 AU/ml) for 5 min. Cells in control experiments received only buffer. Treated (open symbols) and control cells (closed symbols) were subsequently diluted 1000-fold into medium A with 1% sodium pyruvate. At various time intervals samples were plated on CY-agar containing various amounts of NaCl.
△, less than 2×10^{-3}M NaCl;
□, 0.1 M NaCl;
○, 1.0 M NaCl.
The viable count was plotted against the incubation time.

TABLE 2. THE EFFECT OF THE COMPOSITION OF THE MEDIUM AND OF VARIOUS METABOLIC INHIBITORS ON THE RECOVERY OF CELLS INJURED BY STAPHYLOCOCCIN 1580 TREATMENT[a]

recovery medium	log R_{rec} plated on TSA	log R_{rec} plated on TSAS
medium A-pyruvate	0.82	3.67
" succinate	0.10	2.94
" glucose	-0.80	0.40
" -	-0.15	1.90
" pyruvate (anaerobic)	0.70	3.40
phosphate-buffered amino acids + pyruvate[b]	-1.61	1.05
phosphate-buffered amino acids + glucose[b]	-1.54	0.96
30mM phosphate buffer (pH 7.2)	-4.57	<-1
medium A-pyruvate[c]		
+ chloramphenicol	-0.20	0.35
+ actinomycin D	-1.20	0
+ mitomycin C	-0.65	1.54
+ 2,4-dinitrophenol	-1.50	0.05

[a] Washed cells of *S. aureus* Oxford 209P from an exponentially growing culture on TSB, were suspended in potassium phosphate buffer (pH 7.0) in a final concentration of 5×10^8 cells/ml and treated for 5 min at 37°C with 100 AU/ml of staphylococcin 1580. Subsequently samples were diluted 1000-fold into the prewarmed recovery medium under investigation and incubated at 37°C for 2 hours, after which samples were plated on both tryptone soya agar (TSA) and tryptone soya agar supplemented with sodium chloride to a final concentration of 1 M (TSAS) for viability testing.
The logarithm of the survival ratios (R_t) prior to the incubation in the recovery media were -0.80 and -3.90 on TSA and TSAS respectively.
Log R_{rec} is defined as the ratio of the viable count after 2 hours over that immediately after staphylococcin 1580-treatment. The latter was corrected for changes in the viability of cells not treated with staphylococcin 1580, induced by the presence of inhibitors or the incubation conditions.

[b] The medium consisted of 30mM potassium phosphate buffer (pH 7.2); the amino acids listed in Materials and Methods and 0.25% of either glucose or sodium pyruvate.

[c] Final inhibitor concentrations employed were chloramphenicol, 100 µg/ml; actinomycin D, 2 µg/ml; mitomycin C, 0.5 µg/ml; 2,4-dinitrophenol, 0.6 mM.

the effect of specific metabolic inhibitors on the recovery process. Optimal recovery requires a rather rich growth medium. The choice of the main carbon- and energy-source seems to be crucial. Pyruvate, which supports growth of untreated cells very well, was most benefical in the recovery process. Succinate, a less preferable substrate for growth, was also less efective in the recovery process and the omission of an energy source results in a slow recovery. However, glucose, which acts quite well as pyruvate in supporting growth of untreated cells, was not suited at all in sustaining the recovery. Incubation in a glucose medium even improved the killing observed on TSA. Although staphylococcin 1580-treated cells are only slightly impaired as to the rate of glucose uptake (Weerkamp, unpublished observation), they might be blocked in the metabolism of glucose or the energy derived from glycolysis might not be suited for the recovery process. An effect of pH is probably not involved since the internal pH, as determined from the distribution of the weak acid [^{14}C]-5,5-dimethyl-2,4-oxazolidinedion (DMO), is about equal in pyruvate- and glucose-grown cells. Media shown to be suitable for recovery of heat-induced loss of salt tolerance in *S. aureus* (14) did not allow the recovery of cells from staphylococcin 1580-induced injury; this holds for media with various energy sources and buffered by either Tris or phosphate. Incubation in phosphate buffer alone was extremely harmful to staphylococcin-treated cells.

The experiments performed with various metabolic inhibitors show a complete inhibition of recovery by the inhibitors of RNA-synthesis (actinomycin D) and protein-synthesis (chloramphenicol) and by the uncoupler 2,4-dinitrophenol. Also, dicyclohexylcarbodiimide (DCCD), an inhibitor of membrane ATPase, appeared to precent recovery, but the results were obscured by the lethal effect of this agent on cells not treated with staphylococcin 1580. The results obtained with actinomycin D should also be taken with care, since it also might inhibit amino acid transport(11).

Mitomycin C, an inhibitor of DNA synthesis, delayed but did not prevent the recovery. Therefore *de novo* synthesis of RNA

and protein, together with a suitable energy supply, is required to restore the injury induced by staphylococcin 1580.
Preliminary studies (not shown here) on the incorporation of radio-active precursors into macromolecules suggest a specific incorporation of amino acids into membrane proteins during the early stages of the recovery process.

DISCUSSION

Staphylococcin 1580, like colicins E1, K, 1a and 1b and A (5,6,8,19,23) simultaneously inhibits active transport of various substrates and synthesis of macromolecules, induces leak of potassium or preloaded rubidium ions from the cells and declines the ATP level (18). However, as recently shown by Kopecky *et al* (21), these events do not implicate cell death, since plating of colicin E1- or K- treated cells on a medium containing particular concentrations of potassium and magnesium ions allows survival of the cells. Similary, treatment of sensitive cells with staphylococcin 1580 is not simply bactericidal, but cells may survive if (i) the concentration of cations is rather low or (ii) the medium is slightly acidic. Since the presence of salts or alkalinity of the medium during the short incubation of cells with staphylococcin 1580 do not bring about the observed effect on the killing ratio, it is likely that under the described conditions the restoration of the induced injuries is prevented. The observed results may be interpreted on basis of the chemiosmotic hypothesis (10,24).
Extrusion of protons generates a protonmotive force, composed of a pH gradient (ΔpH), inside alkaline, and a membrane potential ($\Delta\psi$), inside negative. At relatively low external pH values ΔpH is large, whereas at neutral and alkaline pH values of the medium $\Delta\psi$ is the most important or sole component. $\Delta\psi$ remains essentially constant at pH range 5-9 (25,27). Since staphylococcin 1580 does not affect the proton-permeability of the membrane (19) it does not abolish pH and, hence, affects the proton motive force less drastically at

low external pH. However, $\Delta\psi$ may be abolished by the movement of permeable cations inverse to the proton extrusion, and hence the generation of the protonmotive force will be prevented at neutral and alkaline pH values. Similar mechanisms have been proposed for the action of the potassium-ionophore valinomycin on both growth of and glutamate uptake by *S. aureus* in the presence of relatively high external potassium concentrations (7,29).

The action of staphylococcin 1580 may induce rather nonspecific membrane permeations, accessible to small cations except protons; the resulting effect on the protonmotive force may be the important factor causing death of the cells. Moreover, the maintenance of a limited internal concentration of potassium ions appears to be of additional importance, since the external presence of potassium ions in addition to sodium ions partly rescuses the cells from killing. Alternatively, the cations might block the recovery at the level of substrate transport. Ring *et al* (28) reported that an almost identical range of cations inhibited amino acid transport in *Streptomyces hydrogenans* by competition at the transport carrier. However, L-glutamate uptake in *S. aureus* was not inhibited by the cations tested, which may object against, although not completely exclude, this possibility.

Rescue of cells treated with colicins E1, or K in media with potassium and magnesium concentrations resembling the intracellular level was reported before (21) and we obtained similar results in studies with colicin A (unpublished result). Cells treated with colicins E1 and K are permeable to potassium ions and behave analogous to valinomycin-treated cells (9). Therefore, a similar mechanism may be involved in the rescue of cells from the injury induced by these various bacteriocins.

Recovery from injury by staphylococcin 1580 appears to be a complex process, requiring at least RNA- and protein-synthesis, and, in addition, metabolic energy derived from a suitable substrate. In many respects the recovery process differs from the well-studied recovery from salt tolerance after heat injury in *S. aureus* (12,14). The latter requires the synthesis of RNA in particular (14) and is accompanied by the res-

toration of the cellular magnesium content (12) and resynthesis of teichoic acid-bound D alanine (13).
Treatment with staphylococcin 1580, however, does not induce gross magnesium leak from the cells (Weerkamp, unpublished result). Preliminary experiments suggest that restoration of the cell damage requires the resynthesis of membrane proteins (unpublished observations). Recently, Knepper and Lusk (20) showed that specific membrane proteins disappeared afer treatment of *E. coli* cells with colicin K or E1.
Although it might be concluded that the action of staphylococcin 1580 is bacteriostatic rather than bactericidal, it should be noted that in most natural environments for staphylococci (e.g. the human skin) salt concentrations are sufficiently high to induce a strong bactericidal effect. Furthermore, recovery requires rather optimal conditions even after very short incubation with the bacteriocin and circumstances disavantageous in this respect enhance the killing ratio strongly.

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CHAPTER 6

PHYSIOLOGICAL CONDITIONS AFFECTING THE SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* TO STAPHYLOCOCCIN 1580

INTRODUCTION

Bacteriocins are widely produced among various bacteria, and some of them, especially colicins, have been studied extensively (22). Bacteriocins from gram-positive bacteria (reviewed recently by Tagg *et al*; 24) are, with a few exceptions, investigated much less thoroughly.

Staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis* 1580 (9,10) exerts its action on the cytoplasmic membrane, and causes a simultaneous inhibition of active transport and macromolecular synthesis, a rapid leak of potassium ions from the cells and a decline of cellular ATP level (11). In this it resembles colicin E1 and K (7) and A (12) and also a variety of bacteriocins from gram-positive bacteria (24).

In a previous study it was shown that sensitive cells are not irreversibly damaged as a consequence of staphylococcin treatment, but the injury may be repaired under appropriate conditions (28). It was also noticed (13) that the staphylococcin 1580-induced killing effect is strongly influenced by the growth temperature of the sensitive cells as well as by the temperature of incubation, which suggests a dependence on the physiological state of the sensitive cells. Similar conclusions were drawn for the action of colicins K (21) and E1 (4) and various bacteriocins from gram-positive bacteria, such as staphylococcin C55 (5) and streptococcin A-FF22 (23).

It was shown that expression of colicin K-action was dependent on a proper fluidity of the membrane (21) and supply of energy (14,19), probably required for the access of the colicin molecule to its target on the cytoplasmic membrane.

In this paper we report on conditions required for the action of staphylococcin 1580 and the role of energy involved in the expression of the killing effect.

Energy may either be derived directly from ATP or drawn from

the high energy state of the membrane (8). According to the chemiosmotic hypothesis (17), a proton-motive force ($\Delta\bar{\mu}_{H^+}$) is created by the extrusion of protons either by oxidation of substrate or by hydrolysis of ATP (8) and is composed of an electrical potential ($\Delta\psi$) and a chemical potential (ΔpH).

MATERIALS AND METHODS

Bacterial strains

Staphylococcin 1580-producing *Staphylococcus epidermidis* 1580 and the indicator strain *S. aureus* Oxford 209P were described previously (9). A staphylococcin 1580-resistant mutant, strain N_3 was derived from the latter by treatment with N-methyl-N'-nitro-N-nitrosoguanidin (1) and selection for staphylococcin 1580 resistance.
The strains were subcultured each fortnight on tryptone soya agar (Oxoid) and stored at 4°C.

Media

Semisynthetic medium A was derived from the previously described AJ-1 medium, except that casein hydrolysate (enzymatically hydrolyzed) was obtained from NBC and either glucose (1% w/v) or sodium pyruvate (1% w/v) was used as carbon source.

Production, purification and assay of staphylococcin 1580

The bacteriocin was prepared and assayed as previously described (28). The activity was expressed in arbitrary units (AU) per ml.

Viable count

Viable counts after staphylococcin 1580-treatment were determined by rapidly diluting samples 1000-fold into ice-cold tryptone soya broth (TSB), and subsequently plating suitable diluted amounts on both tryptone soya agar (TSA) and tryptone soya agar supplemented with 1 M sodium chloride (TSAS) to measure loss of salt tolerance (28). The survival ratio (R_t) was defined as the ratio between the viable counts of treated and untreated bacteria.

Transport of amino acids

Unless otherwise indicated the assay system (0.5 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 50 mM sodium chloride and 2 mM magnesium sulfate (incubation buffer). After 5 min of preincubation at 37°C, the label was added to a final concentration of 10^{-5}M. Samples (0.1 ml) were withdrawn, rapidly filtered on Millipore filter (.45 µ) and washed with 4 ml of the aforementioned incubation buffer. After drying, filters were counted in a liquid scintillation counter.

Determination of the intracellular pH

The intracellular pH was calculated according to Waddell and Butler (25) from the distribution of (^{14}C)-5,5-dimethyl-2,4-oxazolidinedione (DMO) (final cincentration 21.4 µM, 1.0 µCi/ml). Extracellular water was monitored by the use of (^{3}H)-inulin (final concentration 20.0 µM, 1.4 µCi/ml). Aliquots of the incubation mixture were filtered on Millipore filters (0.45 µ) without washing. Filters were dried (15 min, 105°C) and radioactivity was counted in a liquid scintillation counter. Corrections were made for channel efficiency and spill over. The intracellular water space was taken to be 1.55 ml/g dry weight cells (18).

Fluorescence measurements

Fluorescence of 3,3'-dipropyl-thiodicarbocyanine (DiS-C_3) allows the continuous monitoring of the membrane potential (2,16,26). Washed cells were suspended in incubation buffer (pH 7.0) in a standard 1 cm path-length fluorescence cuvet at a final concentration of 50 µg dry weight per ml. DiS-C_3 was added to a concentration of 800 nM and fluorescence was excited at 620 nm and measured at 662 nm by use of an Aminco Bowman spectrofluorimeter with 3 mm slits in the positions 3 and 4. Measurements were carried out at 37°C and the suspensions were aerated through a 28 gauge needle. Scatter due to the bacteria was less than two percent. At the end of each experiment maximum fluorescence of the system was determined after the addition of gramicidin D (1 µg/ml), which allows leveling of H^+, Na^+ and K^+ over the membrane and abolishes the membrane potential comple-

tely. DiS-C_3 did not affect the viability of the cells and did not influence the staphylococcin 1580-induced killing effect.

Fatty acid composition

Extraction and methylation of fatty acids were carried out as described previously (27). Gaschromatograms were run at 170°C on a Pye series 104 gaschromatograph, equipped with 5 ft columns of 10% diethyleneglycolsuccinate (DEGS) on 100-120 mesh Diatomite C.

Pyruvate concentration

The concentration of pyruvate was measured by the method of Dietz (6).

Chemicals

L-(U-^{14}C)-glutamic acid (270 mCi/mmol), L-(U-^{14}C)-glutamine (40 mCi/mmol), L-(U-^{14}C)-proline (290 mCi/mmol), L-(U-^{14}C)-leucine (330 mCi/mmol) and (^{3}H)-inuline (695 mCi/mmol) were obtained from Radiochemical Centre, Amersham, England, (2-^{14}C)-DMO (46.6 mCi/mmol) was supplied by New England Nuclear (Boston, Mass.). Stock solutions of N, N'-dicyclohexylcarbodiimide (DCCD) (Aldrich-Europe, Beerse, Belgium), valinomycin (Calbiochem, Lucerne,Switzerland), gramicidin D and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (both from Boehringer, Mannheim, W.Germany) were prepared in ethanol and stored at -18°C. DiS-C was obtained from Dr. A. Waggoner, Amherst College, Amherst, Mass. Polyethylene sorbitan monooleate (Tween 80) was obtained from Sigma Co. (St. Louis, Miss.). All other chemicals were of reagent grade.

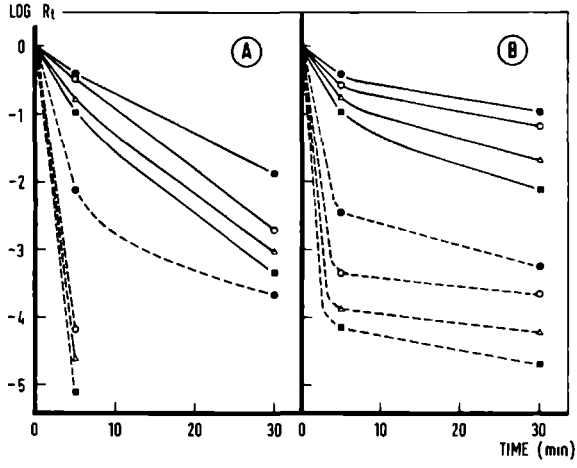


Fig. 1. Effect of the incubation medium on the killing effect of staphylococcin 1580. *S. aureus* Oxford 209P cells from an exponentially growing culture on medium A with 1% sodium pyruvate (1.0×10^9 cells/ml) were treated at 37°C with staphylococcin 1580, either directly in the medium (pH 7.0) or after centrifugation at room temperature, washing and resuspending in incubation buffer, which consisted of 50 mM potassium phosphate (pH 7.0), 50 mM sodium chloride and 2 mM magnesium sulfate. Samples were withdrawn after 5 and 30 minutes and plated on both tryptone soya agar (TSA, solid lines) and TSA supplemented with 1 M sodium chloride (TSAS, broken lines). Viable counts are expressed as the ratio of staphylococcin 1580 treated and untreated suspensions (R_t).
●, 10 AU/ml; ○, 40 AU/ml; △, 100 AU/ml; ■, 250 AU/ml of staphylococcin 1580.
(A) Cells in medium A-pyruvate. (B) Cells in incubation buffer.

RESULTS

Composition of the incubation medium

Staphylococcin 1580 appears to be more lethal to cells treated in growth medium than to washed cells suspended in a simple buffer medium (Fig. 1). This is evident when treated cells are plated on TSA as well as for cells plated on the same medium containing 1 M sodium chloride. The viable count on the latter medium is much lower due to the inhibiting effect of sodium chloride on the rescue of cells from the primary injury induced by the staphylococcin (28). The different effect exerted by the

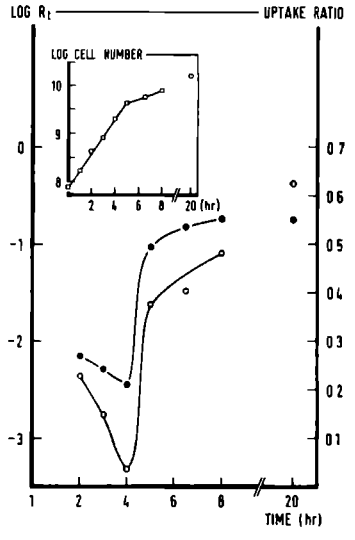


Fig. 2. Relation between the growth phase of the indicator cells and their sensitivity to staphylococcin 1580. An overnight culture of *S. aureus* Oxford 209P was inoculated 1:200 into fresh medium A containing 1% sodium pyruvate at 37°C. At intervals samples were taken to determine the viable count and aliquots were diluted with fresh, prewarmed medium, to give about 5 x 10^8 cells/ml. Staphylococcin 1580, dissolved in 50 mM potassium phosphate buffer (final pH 7.0, concentration 100 AU/ml), or only buffer were added, immediately followed by (U-^{14}C)-L-glutamic acid (final concentration 2µCi/ml) and the mixture was incubated for 15 min at 37°C. Subsequently viability was determined by plating on TSA and total uptake of label by filtering aliquots and washing with 5 ml of medium A. Viability is given as the survival ratio (R_t) and the uptake of glutamic acid as ratio of dpm between treated and untreated cells. ●, viability; ○, glutamic acid uptake. Insert:□ , growth curve.

growth medium and the buffer did not result from the different handling of the cells during the wash and resuspension procedure, since similarily treated cells, washed with and resuspended in growth medium were killed to the same extent as cells not handled in this way. Moreover, addition of various energy sources, like glucose, pyruvate and L-lactate, to the buffer medium did not lower the survival ratio. The different effects can neither be explained on basis of the presence of salts in the buffer, since salts affect the staphylococcin 1580-induced killing only slightly (28). Also pH values between 5.5 and 8.5 have only minor influence; a distinct optimal killing effect around pH 6.5 could, however, be observed (28). At one hand the maximal number of killed cells was largely reached after about 5 min with cells treated in buffer, but cells treated in growth medium are progressively killed after that time interval. This may suggest

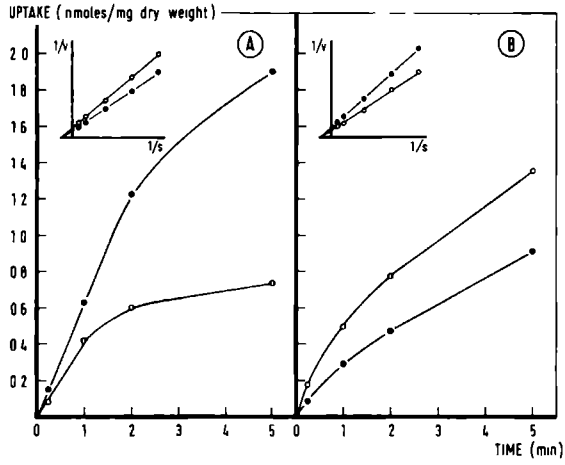


Fig. 3. Effect of staphylococcin 1580 on the uptake of glutamic acid in cells grown with either glucose or sodium pyruvate. Mid-exponential phase cells, growing in medium A with either 1% pyruvate or 1% glucose, were centrifuged, washed and resuspended in incubation buffer (pH 7.0), to a final concentration of 0.25 mg dry weight cells per ml. 0.5 ml of cell suspension was preincubated for 5 min at 37°C after which staphylococcin 1580 (final concentration 100 AU/ml) or buffer were added, followed after 1 min by (U-^{14}C)-L-glutamic acid to 10^{-5}M final concentration. Samples (100 µl) were removed at intervals, filtered and washed with 4 ml incubation buffer to determine the radioactivity. To determine the Km-values, various final concentrations of glutamic acid were applied and the uptake was measured after 30 seconds. ●, treated with staphylococcin 1580 in buffer; ○, treated with buffer only. (A) Cells grown with glucose; (B) cells grown with sodium pyruvate. The log R_t-value, determined by plating on TSAS after 5 min of staphylococcin 1580-treatment was - 2.45 and - 2.32 for pyruvate- and glucose-grown cells, respectively.

that only a part of the buffer-suspended cells is sensitive, whereas in growth medium the temporarily insensitive cells enter sensitive stage progressively.

Influence of growth phase

Both the survival ratio and the inhibition of glutamic acid uptake after staphylococcin 1580-treatment are strongly dependent

on the growth phase in batch cultures of the tested cells (Fig. 2). Cells growing in medium A with sodium pyruvate as source of energy are optimally sensitive in the mid-exponential phase. A sudden change in sensitivity occurs coincidently with an alteration of the growth rate. At this time the pyruvate concentration in the medium still amounts to over 80% of the initial value; growth continues for at least another two generations and ceases when pyruvate is completely used up.

Effect of the energy source

The effect of the main carbon- and energy-source in the growth medium on the sensitivity of cells to staphylococcin 1580 was investigated in order to elucidate the possible involvement of distinct catabolic pathways in the staphylococcin action. Although both glucose- and pyruvate-grown cells are killed by the bacteriocin to the same extent when plated on TSAS, pyruvate-grown cells are apparently damaged more severely, since the survival ratio on TSA is considerably smaller: log R_t is -0.22 and -1.15 for glucose- and pyruvate-grown cells, respectively, after 5 min of staphylococcin treatment. This is particularily clear when the effect of the bacteriocin on uptake of glutamic acid (Fig. 3) or on the membrane potential (Fig. 4) is studied. Uptake in pyruvate-grown cells is inhibited by staphylococcin 1580, but in contrast, it is stimulated in glucose-grown cells. In both cases the K_m for transport, which amounts to 1.2×10^{-4}M, was not affected, which is in accordance with the previous reported results, obtained with membrane vesicles (12). Similar observations were made with the amino acids leucine, proline and glutamine.

The effect of various inhibitors on the glutamic acid uptake in both types of cells is shown in Table 1. Uptake is strongly inhibited by the uncoupler FCCP and by valinomycin in the presence of external potassium ions, which confirms the previous conclusion on its coupling to a high energy state of the membrane (12). DCCD, an inhibitor of the membrane-bound Ca^{2+}, Mg^{2+} - ATPase exhibits a divergent effect: uptake in glucose-grown cells is inhibited more severely than in pyruvate-grown cells at moderate inhibitor concentrations. This suggests that energy

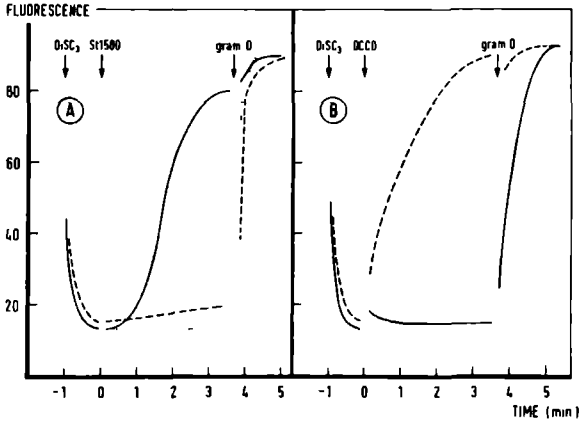
TABLE 1. EFFECT OF INHIBITORS ON GLUTAMIC ACID UPTAKE IN *S. AUREUS* OXFORD 209P GROWN WITH GLUCOSE OR PYRUVATE[a]

Inhibitor		Glutamic acid uptake			
		Glucose-grown cells		Pyruvate-grown cells	
		nmoles/mg dry weight/min	% of control	nmoles/mg dry weight/min	% of control
None		0.28	100	0.62	100
DCCD	10^{-5}M	0.15	54	0.60	97
	4×10^{-5}M	0.13	45	0.68	110
	10^{-4}M	0.08	30	0.22	36
	2.5×10^{-4}M	0.02	8	0.05	6
Valinomycin	10^{-6}M	0.12	44	0.22	36
FCCP	10^{-6}M	0.03	11	0.04	7
KCN	10^{-2}M	0.05	18	0.06	9

[a] Preparation of cells (0.25 mg dry weight per ml) and uptake of glutamic acid is described in the legend to Fig. 3. Uptake was measured 30 sec after addition of the label. Inhibitors were added in the final concentrations indicated. Valinomycin and FCCP were added 1 min, DCCD and KCN 10 and 15 min, respectively, prior to the addition of the label. Except for KCN, the inhibitors were dissolved in ethanol. The final ethanol concentration never exceeded 1% which did not affect the glutamic acid uptake.

used for glutamic acid uptake in glucose-grown cells is at least in part coupled by way of the membrane ATPase, whereas in pyruvate-grown cells the oxidative input through electron transport is relatively more important.

These findings are confirmed in the studies with the fluorescent probe DiS-C_3, recently used by several investigators to measure the electrical potential ($\Delta\psi$) across the membrane (2,15,16,26). Preliminary experiments showed that this technique properly reflects the changes in the membrane potential expected from valinomycin-treatment (1µg/ml) of *S. aureus* cells and membrane



Effect of staphylococcin 1580 and DCCD on DiS-C_3 fluorescence. Cells were grown and prepared as described in Fig. 3, except that the final concentration of cells was 50 µg dry weight per ml. Suspensions (2,5 ml) were preincubated in the cuvette at 37°C for 5 min, after which 10 µl of DiS-C_3 (final concentration 800 nM) were added. After 1 min either staphylococcin (final concentration 25 AU/ml) or DCCD (final concentration $5x10^{-5}$ M) was admixed. At the end of the experiment gramicidin D (final concentration 1 µg/ml) was added to determine the maximal fluorescence. Full lines represent *S. aureus* 209P cells grown with glucose; dotted lines, *S. aureus* N3 cells grown with sodium pyruvate. The log R_t-values, as determined by plating on TSAS, were - 2.10 and - 2.38 for *S. aureus* Oxford 209P grown on glucose and sodium pyruvate, respectively, and + 0.05 for *S. aureus* N3. (A) Effect of staphylococcin 1580. (B) Effect of DCCD.

vesicles, suspended in either sodium phosphate buffer (hyperpolarization) or potassium phosphate buffer (depolarization) under the conditions applied in the present experiments. Both glucose-grown and pyruvate-grown cells exhibited about identical levels of fluorescence quenching and, hence, possess a similar membrane potential (Fig. 4). DCCD did not affect the membrane potential in pyruvate-grown cells, but depolarized glucose-grown cells largely (Fig. 4B), which corroborates the different way of energy coupling. Staphylococcin 1580 had a pronounced effect on the membrane potential of pyruvate-grown cells (Fig. 4A): after a 20 seconds lag the fluorescence quen-

TABLE 2. EFFECT OF STAPHYLOCOCCIN 1580 ON THE INTRACELLULAR pH OF CELLS GROWN WITH EITHER GLUCOSE OR SODIUM PYRUVATE

Time after addition of staphylococcin 1580 (min)	Internal pH	
	Glucose-grown cells	Pyruvate-grown cells
1	7.78	7.53
5	7.74	7.58
10	7.65	7.55
15	7.62	7.61
No staphylococcin 1580	7.65 ± 0.03	7.65±0.02

Cells from exponentially growing cultures on either medium A-glucose or medium A-pyruvate, were centrifuged, washed and resuspended (0.45 mg dry weight per ml) in 50 mM potassium phosphate buffer (pH 7.00), and incubated at 37°C. (^{3}H)-inuline (20 µM, 1.4 µCi/ml) and (^{14}C)-DMO (21.4 µM, 1.0 µCi/ml) were added, immediately followed by staphylococcin 1580 (final concentration 100 AU/ml). Samples taken at intervals were filtered without washing and the radioactivity was counted. After 15 min the incubation mixture was centrifuged and 10 µl aliquots of the supernatant were counted to determine the radioactivity in the external liquid.

ching is partly abolished, most probably due to a partial depolarization of the membrane potential. Increasing amounts of staphylococcin 1580 did not further abolish the membrane potential, nor did it affect the length of the lag time. In contrast, the membrane potential of glucose-grown cells is affected only slightly by staphylococcin 1580-treatment. Further addition of staphylococcin 1580 did not enhance the response. The membrane of the staphylococcin 1580-resistant strain N3, grown either on glucose or pyruvate, was not affected by the bacteriocin.

In a previous paper it was shown by use of the acid-pulse technique that the permeability of the membrane to protons was not altered by treatment with staphylococcin 1580 (12). Table 2 shows the intracellular pH values for glucose- and pyruvate-grown cells in the presence or absence of staphylococcin 1580, as calculated from the distribution of the weak acid (^{14}C)-DMO.

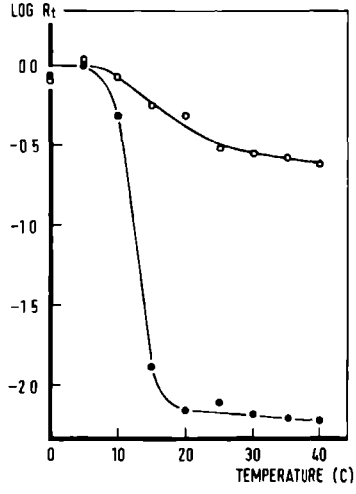


Fig. 5.
Effect of the incubation temperature on staphylococcin 1580-induced killing. *S. aureus* Oxford 209P was grown at 37°C into mid-exponential phase on tryptone soya broth (TSB), centrifuged, washed and resuspended in incubation buffer (pH 7.0). Subsequently cells were preincubated for 5 min at various temperatures, after which staphylococcin 1580 (final concentration 100 AU/ml) or only buffer was added and the mixtures were incubated further for 5 min. Samples were removed, rapidly diluted 1000-fold into ice-cold TSB and plated on either TSA (○) or TSAS (●). The viability, expressed as the survival ratio (R_t), is plotted against the temperature of incubation.

The intracellular pH, and, hence, the pH-gradient (ΔpH) over the cytoplasmic membrane at constant external pH, is identical in glucose- and pyruvate-grown cells, and remained constant throughout the incubation period. Incubation with staphylococcin 1580 had only a slight effect: a small and transient rise of the internal pH can be observed in glucose-grown cells, whereas the internal pH of pyruvate-grown cells was temporarily lowered.

Effect of temperature

The extend of staphylococcin 1580 induced injury, as determined by culturing treated cells on high-salt (TSAS) medium, is strongly dependent on the incubation temperature applied during the treatment with the bacteriocin (Fig. 5). A transition from

TABLE 3. EFFECT OF CULTURE CONDITIONS ON THE TEMPERATURE PROFILE OF STAPHYLOCOCCIN 1580-INDUCED LOSS OF SALT TOLERANCE

Growth temperature (°C)	Transition temperature (°C)	log R_t
22	14	-3.5
29	12	-2.8
37	12	-2.2
42	14	-2.4
37 (10% Tween 80)	6	-3.0

Cells were grown in TSB, prepared and treated with staphylococcin 1580 (final concentration 100 AU/ml) as described in the legend to Fig. 5, except that the indicated growth temperatures were applied. In one experiment the growth medium contained 10% (v/v) Tween 80. Viable counts were determined on TSAS and expressed as the survival ratio (R_t). The transition temperature is defined arbitrarily as the temperature at which the survival ratio was 10%, and was calculated from curves similar to that shown in Fig. 5.

resistance to full sensitivity, takes place over a relalively small temperature range, which suggests that a conformational change occurs either in the staphylococcin molecule, or in the indicator cells. Attempts to demonstrate a conformational change in the staphylococcin molecule by use of the fluorescent probe 1-anilino-8-naphtalene sulfonate (ANS^-) were unsuccessful. A variation of the growth temperature over a range from 22 to 42°C affected mainly the proportion of the cells sensitive to the bacteriocin and only slightly the temperature profile observed in the sensitivity test (Table 3). Cells cultured at either 37°C or 22°C differed only slightly with respect to their fatty acid composition (Table 4). However, the transition temperature range was decreased in cells cultured in the presence of Tween 80, which greatly enhanced the proportion of unsaturated fatty acids (Table 4). Therefore, the transition temperature may reflect changes in the fluidity of the membrane lipids.

TABLE 4. EFFECT OF GROWTH TEMPERATURE AND OF TWEEN 80 ON THE FATTY ACID COMPOSITION OF *S. AUREUS* OXFORD 209P

Fatty acid	Percentage of total fatty acids		
	37°C	22°C	37°C + Tween 80
br-C13:0	0.7	0.5	0.5
n-C13:0	0.5		1.1
br-C14:0	2.6	2.4	6.7
br-C15:0	38.0	42.9	33.5
n-C16:0	8.6	8.2	6.3
br-C17:0	6.0	3.8	3.1
n-C18:0	17.6	15.5	10.7
n-C18:1	1.4	4.3	17.3
br-C19:0	0.7	1.0	1.0
n-C19:0	8.1	4.7	4.9
n-C20:0	8.2	9.8	6.1
n-C20:1	0.5	1.2	7.4
total branched	51.6	53.6	45.5
total unsaturated	1.9	5.5	24.7
main chain length (number of carbon atoms)	15.6	15.7	16.7

Cells were grown in TSB or TSB supplemented with 10% (^{v}/v) Tween 80 at the temperature indicated into mid-exponential phase and washed twice with 50 mM potassium phosphate buffer (pH 7.0). Fatty acids were extracted as described in Materials and Methods. Minor fractions, containing less than 0.5% of the total fatty acids, are not shown. Branched chain fatty acids are designed br, straight chains n.

The effects of staphylococcin 1580 on sensitive cells, such as killing, inhibition of amino acid transport and abolition of the membrane potential, require distinct physiological conditions of the cells for full expression. It must be noted here, that energy is required to induce any physiological damage since energy-depleted cells are entirely resistant to the bacteriocin (Chapter 7). Cells pregrown with either glucose or sodium pyruvate as carbon- and energy-source differed as to the extent of primary injury induced by staphylococcin, but they were equally sensitive to killing by high concentrations of salt after treatment with the bacteriocin. The primary injury was much more severe in pyruvate-grown cells as was obvious from its expression in the extent of irreversible killing, the inhibition of active transport and the depolarization of the membrane potential.
The effects of DCCD indicate that the membrane energization results from ATP hydrolysis by the Ca^{2+}, Mg^{2+} - ATPase in glucose-grown cells, whereas an electron transport-coupled membrane potential is predominant in pyruvate-grown cells. These results suggest that staphylococcin 1580 acts primarily on a target specifically involved in the maintenance of the membrane potential coupled to electron transport. However, the electron transport and the function of dehydrogenases were not inhibited (12).
Staphylococcin 1580 abolished the membrane potential only partly. Brewer (3) suggested recently that only part of the sources of the membrane potential are blocked by colicin K in *E. coli*. A similar conclusion may be valid for the action of staphylococcin 1580.
An exponentially growing cell population was heterogeneous with respect to staphylococcin sensitivity. The rapid shift in the proportion of sensitive cells during exponential growth may be due to a change in energy metabolism of the cells.
Both the growth temperature of the indicator cells and the incubation temperature during staphylococcin treatment affected the sensitivity of the cells to bacteriocin. Cells were insensitive to staphylococcin when incubated with it at a temperature

below a rather sharply defined limit. The access of staphylococcin to its target may be prevented at low temperature or the expression of its effect may be blocked. The action of colicin K depends also strongly on the temperature. The effect was attributed to changes in the membrane lipids affecting the fluid properties of the membrane (19,21). This may be valid also for the effect of temperature on cells treated with staphylococcin 1580, since the temperature at which cells become sensitive was lower in cells containing increased levels of unsaturated fatty acids.

Cells grown at suboptimal temperatures were killed to a greater extent when cultured in the presence of high concentrations of salt after the staphylococcin treatment, than cells grown at the optimal temperature. This result seems to contradict previous ones, since cells grown at suboptimal temperatures were killed to a less extent than those grown at optimal temperatures, when cultured on normal media after treatment with staphylococcin (13). This discrepancy may be explained by assuming effects of the growth temperature on the ability of the cells to repair injury after staphylococcin treatment (28), as well as, on the extent and expression of the primary injury caused by the bacteriocin.

Various degrees of injury may be observed in cells treated with staphylococcin 1580, but they reflect distinct physiological conditions of the cell. Our data do not justify the distinction of the action of staphylococcin 1580 into successive stages, as was possible for the action of various colicins (20). Staphylococcin-treated cells were not rescuable by trypsin (13) and physiological injury occurred in the very early stages of treatment. Effects exerted by the energy metabolism of the cell and by the temperature are similar to those found with colicin E1 and K during the transition step from stage I to stage II (14, 19,21).

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CHAPTER 7

ENERGY REQUIREMENT FOR THE ACTION OF STAPHYLOCOCCIN 1580 IN *STAPHYLOCOCCUS AUREUS*

INTRODUCTION

Staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis* 1580, affects primarily the maintenance of the energized state of the membrane in sensitive cells (30), and its action results in inhibition of active amino acid transport (11), leakage of ions from the cells (11), a decrease of intracellular ATP-level (11), inhibition of macromolecular synthesis (11), and loss of salt tolerance and viability (31). However, the permeability to protons (12,30) and the uptake of α-methylglucoside (32) are not affected, which shows that the general integrity of the permeability barrier of the cells remains intact.

Previous experiments had shown that the effect induced by staphylococcin 1580 in sensitive cells is dependent on the physiological state, particularly the energy metabolism, of the cells (30). Recently, it was shown that the initation of colicin action in *Escherichia coli* required a high energy state of the membrane, in the absence of which the cells remained rescuable by trypsin (14).

In the present study we investigated the ability of various carbon sources to induce sensitivity to staphylococcin 1580 in cells starved for endogenous energy sources, and the effect of various conditions on the reenergization process.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Staphylococcin 1580-producing *Staphylococcus epidermidis* 1580 and the indicator strain *S. aureus* 209P were described previously (30). Mutant strains of the latter were derived by ultraviolet irradiation: strain M17 (men^-) was enriched in the presence of kanamycin and selected for growth stimulation by menadione

as described by Tien and White (29); strain SA4 was selected for inability to grow on nonfermentable substrates as described by Daniel *et al* (8). The strains were subcultured each fortnight on tryptone soya agar (Oxoid), supplied with menadione (2 μM) in the case of strain M17, and stored at 4°C.
The bacteria were grown aerobically at 37°C in semi-synthetic medium A (30) either with glucose (1%) or sodium pyruvate (1%) as the carbon source, except that strain M17 was grown in medium AN, which was identical to medium A with 1.0% glucose as carbon source supplied with 0.25% sodium pyruvate and 5 mg/l uracil, either with or without menadione (final concentration 6 μM). Anaerobic cultures were grown in tightly stoppered 100 ml serum flasks, filled with 50 ml oxygen-free medium AN under an argon atmosphere. For the induction of nitrate reductase, 25 mM $NaNO_3$ was added. The bacteria were harvested in mid-exponential phase of growth, washed twice with incubation buffer, containing 50 mM potassium phosphate (pH 7.0), 50 mM sodium chloride and 10 mM magnesium sulfate, and finally resuspended in the same buffer.

Starvation procedure
Cells were starved for endogenous energy reserves by a modification of the method described by Berger (2). Washed cells (absorption at 600 nm, A_{600} 1.5) were incubated for 2 h at 37°C with vigorous aeration in the aforementioned incubation buffer, supplied with 2,4-dinitrophenol (final concentration 0.8 mM, unless otherwise indicated). Subsequently, the cells were washed 4 times and resuspended in incubation buffer.

Anaerobic studies
In anaerobic experiments oxygen-free nitrogen was bubbled through the incubation mixture, either in the presence or absence of 1 mM sodium dithionite. Anaerobiosis was routinely checked in control suspensions that received in addition 0.001% benzyl viologen (-0.359V).

Production and assay of staphylococcin 1580

The bacteriocin was prepared and assayed as previously described (30). The activity was expressed as arbitrary units per ml.

Viable count

Viable counts after staphylococcin 1580-treatment were determined by rapidly diluting samples 1000-fold into ice-cold tryptone soya broth, and subsequently plating suitably diluted amounts on both tryptone soya agar (TSA) and TSA supplemented with 1 M NaCl (TSAS) to measure the loss of salt tolerance (31). The survival ratio (R_t) was defined as the ratio between the viable counts of treated and untreated suspensions.

Uptake of L-glutamic acid

The uptake of L-[U-^{14}C]glutamic acid (270 mCi/mmol, 1.7 µM final concentration) was measured as previously described (30).

Measurement of ATP

Extraction and assay of ATP by use of the luciferin-luciferase system was described previously (11), except that the final volume in the scintillation vial was reduced to 1.0 ml.

Measurement of nicotinamide nucleotides

After the incubation the cells were concentrated by centrifugation for 2 min at 18.000 x *g*. The pellets were resuspended either in 1 ml 0.2 M $HClO_4$ in 50% ethanol to extract NAD^+ and $NADP^+$, or in 1 ml 0.2 M KOH in 50% ethanol to extract NADH and NADPH, and were incubated for 15 min at room temperature, chilled in an ice-bath and neutralized with 3N KOH and 3N HCl, respectively.

The concentrations of nicotinamide nucleotides were measured immediately thereafter by use of the enzymatic cycling method described by Nisselbaum and Green (21).

Measurement of potassium leak

The potassium concentration in the external medium was measured by flame photometry after removal of the cells by centrifugation for 2 min at 18.000 x *g*.

Oxygen consumption

The uptake of oxygen was measured by manometric technique using a Gilson respirometer, according to the recommendations of the manufacturer.

Chemicals

L-[U-^{14}C]glutamic acid (270 mCi/mmol) was obtained from Radiochemical Centre, Amersham, England. Kanamycin sulfate and neomycin sulfate were from Boehringer, Mannheim, W. Germany; 2,4-dinitrophenol was from Baker, Deventer, The Netherlands; cephalexin was from Glaxo Lab. Ltd, Greenford, England; and dried firefly lantern extract from Sigma Co, St Louis, Missouri. Stock solutions of N,N'-dicyclohexylcarbodiimide (DCCD) (Aldrich-Europe, Beerse, Belgium), valinomycin (Calbiochem, Lucerne, Switzerland) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Roehringer, Mannheim, W. Germany) were prepared in ethanol and stored at -18°C. Chloropyruvate was prepared by M.H. de Jong from this department, according to the method of Cragoe and Robb (6), purified by vacuum sublimation, stored desiccated at -80°C and dissolved immediately before use. All other chemicals were of reagent grade.

RESULTS

The effect of the carbon source

S. aureus cells, grown with glucose and subsequently starved to reduce the endogenous energy reserves, remain completely viable upon treatment with staphylococcin 1580 (Fig. 1). Incubation with a suitable carbon source rapidly restores the sensitivity (Table 1). The loss of salt tolerance, as tested on TSAS plates, in the presence of various carbon sources follows first-order kinetics, but the slope depends on the kind of substrate applied (Fig. 1). It should be noted that the sensitivity to staphylococcin 1580 does not correspond directly to the "energy-charge" of the cells. The latter was estimated from the intracellular ATP level and from the energized state of the membrane, as reflected in the rate of L-glutamic acid uptake which is directly coupled to it (12,30). Starved cells, incubated with

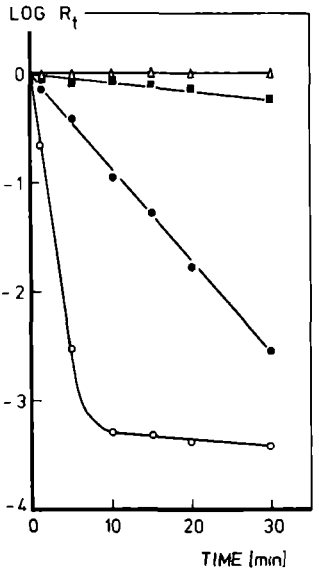

Fig. 1. Effect of carbon source on the staphylococcin 1580-induced loss of salt tolerance in starved *S. aureus*. Cells were aerobically grown with glucose and starved for endogenous energy reserves. The cells were suspended ($6x10^8$ cells/ml) in 50 mM potassium phosphate (pH 7.0), 50 mM sodium chloride and 10 mM magnesium sulfate, and incubated at 37°C with staphylococcin 1580 (100 arbitrary units per ml) in the presence of 20 mM glucose (○), 10 mM L-lactate (●), 10 mM D-lactate (■) or without a carbon source (Δ). Samples were taken at intervals and viable counts were made by plating on TSAS. The viable counts are expressed as the logarithm of the survival ratio.

L-lactate, which can directly serve as a donor of electrons to the respiratory chain and induces active uptake of amino acids (28), have consistently higher levels of ATP and show increased rates of L-glutamic acid uptake as compared to cells energized with glucose. However, glucose induces staphylococcin 1580-sensitivity much more efficiently (Table 1). This discrepancy is even more pronounced when the incubation time of the starved cells with the carbon source prior to the addition of the bacteriocin is varied (Fig. 2). No major influence of the prolonged incubation time was found on the abilities of glucose to stimulate sensitivity or to stimulate L-glutamic acid uptake. However, when L-lactate is applied, the cells are initially relatively sensitive to the bacteriocin, but after about 5 min of incubation a sudden break to almost complete insensitivity occurs. Simultaneously, a strong increase in the ability to transport L-glutamic acid takes place. The rate of oxygen consumption remains unaltered during the entire incubation period (not shown). This suggests that the sensitivity to staphylo-

TABLE 1. EFFECT OF STAPHYLOCOCCIN 1580 ON THE SURVIVAL OF STARVED CELLS OF *S. AUREUS* IN THE PRESENCE OF VARIOUS CARBON SOURCES

Growth medium	Carbon source (mM)		log survival ratio at 5 min: cells plated on TSA	log survival ratio at 5 min: cells plated on TSAS	ATP-concentration (nmol/mg dry wt)	L-glutamic acid uptake (nmol/min per mg dry wt)	Oxygen-consumption (µmol/min per mg dry wt)
A-glucose	none		0.01	-0.02	0.6	0.07	0.02
	glucose	20	-0.85	-2.60	6.7	0.45	0.12
	α-methylglucoside	20	0.02	0.0	-	-	-
	fructose	20	-0.68	-2.35	-	-	-
	α-glycerophosphate	10	-0.10	-0.42	2.6	0.25	0.07
	3-phosphoglycerate	10	0.0	-0.21	1.1	0.21	-
	pyruvate	10	0.04	-0.10	2.9	0.10	0.02
	D-lactate	10	-0.06	-0.12	-	-	0.04
	L-lactate	10	-0.08	-0.92	9.2	0.72	0.40
	citrate	10	0.05	0.0	0.9	0.12	-
	succinate	10	0.0	-0.05	2.9	0.50	-
A-pyruvate	none		-0.71	-1.90	0.4	0.03	0.04
	glucose	20	-1.10	-2.74	4.2	0.23	0.17
	pyruvate	10	-0.95	-2.47	7.2	-	-
	L-lactate	10	-1.43	-2.94	6.8	0.54	0.56

Table 1. Suspensions of starved cells ($6x10^8$ cells per ml) pregrown in medium A with either glucose or pyruvate were prepared as described in Materials and Methods, and incubated for 5 min at 37°C with the carbon sources listed in the Table. Then, samples were removed to measure the intracellular ATP level; the remainder received staphylococcin 1580 (100 arbitrary units per ml) and was incubated for another 5 min, after which the survival and loss of salt tolerance were measured by plating on TSA and TSAS, respectively. L-glutamic acid uptake (1.7 μM, 270 mCi/mmol) and oxygen consumption were measured in separate experiments under identical conditions, after 5 min of preincubation with the carbon source tested.
- not tested.

TABLE 2. CONCENTRATION OF NICOTINAMIDE NUCLEOTIDES IN STARVED AND RECONSTITUDED CELLS OF *S. AUREUS*

Growth medium	Energy source	Concentration of NADH + NAD^+ (nmol/mg dry wt)	NADH/NAD^+ ratio
A-glucose	(not starved)	5.20	0.30
A-glucose	none	1.54	0.17
	glucose	1.56	0.46
	L-lactate	1.54	0.31
	D-lactate	1.49	0.21
A-pyruvate	none	1.88	0.16
	glucose	2.50	0.75

The experimental conditions were identical to those used in Table 1. After 5 min of incubation at 37°C with the indicated carbon source, the cells were collected by centrifugation for 2 min at 18.000 x *g* and extracted as described in the Materials and Methods section.

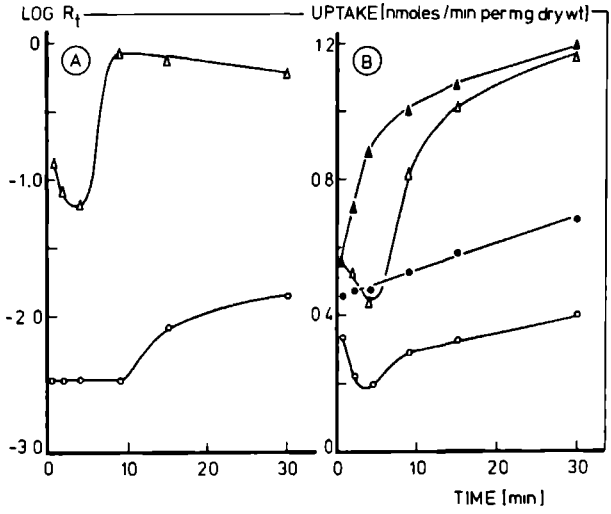

Fig. 2. Influence of the incubation time with glucose or L-lactate on the action of staphylococcin 1580. Cells were aerobically grown with glucose, starved for endogenous energy reserves and incubated (8×10^8 cells/ml) at 37°C with either 20 mM glucose (circles) or 10 mM L-lactate (triangles) for various times prior to the addition of staphylococcin 1580 (140 arbitrary units per ml) or the radioactive label.
A. The viability was determined 5 min after the addition of staphylococcin 1580 by plating on TSAS. Δ, L-lactate; 0, glucose.
B. The uptake of L-glutamic acid (1.7 µM, 270 mCi/mmol) was measured 1 min after the addition of staphylococcin 1580 and the label. Open symbols: in the presence of staphylococcin 1580; closed symbols: no staphylococcin present.
Δ,▲, L-lactate; 0,●, glucose.

coccin 1580 is in some way connected to the energized state of the cell. However, the extent of staphylococcin 1580-induced effects does not directly reflect the energized state of the cell, but rather depends on the route of energy flow in the cell.

The inefficacy of most of the other substrates tested, is due to the failure of starved cells to take up these substrates, or their inability to serve as an electron donor to the respiratory chain in these cells.

In contrast to cells grown with glucose as carbon source, starved cells that were pregrown with pyruvate remain partially sensitive to the bacteriocin in the absence of an exogenous carbon source (Table 1), despite of a strongly reduced energy charge. Aggravation of the starvation procedure by increasing its duration or the 2,4-dinitrophenol concentration up to 10mM, did not decrease the basal sensitivity, but finally resulted in the inability to enhance the killing effect after addition of exogenous energy sources (results not shown).

Since nicotinamide nucleotides occupy a central place in energy-transforming and oxidation-reduction reactions, and probably in their regulation, we investigated the levels of NADH and NAD^+ in starved and reconstituted cells (Table 2). Gererally, bacteria maintain a rigid level of NADH rather than a constant ratio of the reduced to the oxidized form (20,33). In our results the total amount of NADH + NAD^+ was strongly reduced in starved cells when compared to non-starved cells, but was fairly constant in starved cells under the tested conditions. The values found for non-starved cells are similar to those reported for *S. albus* (33). The relative amount of the reduced compound was low in starved cells, but increased upon reenergization. It should be noted, that the NADH/NAD^+ ratio was significantly higher when glucose was applied than with L-lactate as the energy source. It is not likely that the lower ratio in L-lactate-stimulated cells is the result of anaerobic conditions that could arise during the centrifugation step, since anaerobic conditions applied in experiments with *S. albus* did not influence the amounts of nicotinamide nucleotides during short periods (33). The results in Table 2 suggest that the redox-state of the cell could be related to the restoration of the staphylococcin 1580 sensitivity. However, in starved, pyruvate-grown cells also low amounts of NADH were found, although these cells are partially sensitive to the bacteriocin.

The effect of metabolic inhibitors

Table 3 shows the effects exerted by various metabolic inhibitors on the reenergization and staphylococcin 1580-sensitivity in starved cells incubated with either glucose or L-lactate.

TABLE 3. THE EFFECT OF INHIBITORS OF THE ENERGY METABOLISM ON THE ACTION OF STAPHYLOCOCCIN 1580 IN STARVED AND RECONSTITUTED CELLS OF *S. AUREUS*

Carbon source	Inhibitor		log survival ratio at 5 min (plated on TSAS)	ATP-concentration (nmol/mg dry wt)	%	L-glutamic acid uptake (nmol/min per mg dry wt)	%
none	none		-0.10	0.1		0.06	
glucose	none		-2.42	6.7	100	0.42	100
	KCN	10 mM	-0.60	0.8	11.8	0.07	16.5
	azide	10 mM	-1.35	-	-	-	-
	arsenate[a]	20 mM	-0.08	1.6	23.8	0.13	31.0
	FCCP	2 µM	-0.10	<0.1	< 1	0.01	2.5
	valinomycin	2 µM	-0.40	1.4	20.9	0.04	9.5
	DCCD	50 µM	-2.20	6.3	94.0	0.05	12.0
	iodoacetate	1 mM	0.05	<0.1	< 1	-	-
	chloropyruvate	5 mM	0.05	<0.1	< 1	-	-
L-lactate	none		-0.86	8.8	100	0.68	100
	KCN	10 mM	0.05	0.4	4.5	0.01	1.5
	azide	10 mM	-0.28	4.4	50.0	-	-
	arsenate[a]	20 mM	-0.17	3.4	38.6	0.66	97.2
	FCCP	2 µM	-0.08	0.2	2.2	0.01	1.5
	DCCD	50 µM	-0.31	3.8	43.2	0.42	61.8
	iodoacetate	1 mM	-0.88	7.9	89.8	0.70	102.5
	chloropyruvate	5 mM	-0.20	3.2	36.4	0.30	44.2

Table 3. Cells were grown aerobically with glucose, starved for endogenous energy reserves, and incubated at 37°C for 5 min without an exogenous energy source. Then the inhibitors listed in the Table were added, immediately followed by either glucose (20 mM) or L-lactate (10 mM) and the incubation was continued. After 5 min samples were removed to measure the intracellular ATP level and the remainder was split into two portions, one of which received staphylococcin 1580 (100 arbitrary units per ml). 5 min later the viable count was measured by plating on TSAS. The uptake of L-glutamic acid (1.7 µM, 270 mCi/mmol) was measured in separate experiments under identical conditions. The label was added 5 min after the inhibitors and the carbon source had been applied, and the uptake was measured one min later.

[a]Incubations with arsenate were carried out with 50 mM Tris·HCl (pH 7.0) replacing the potassium phosphate in the incubation medium. Replacement of buffer did not affect the values in experiments without arsenate.

Inhibitors of glucose uptake, such as chloropyruvate (15), or glycolysis, such as iodoacetate, completely block the initiation of the sensitivity to staphylococcin 1580 and ATP-formation with glucose, but not with L-lactate. Arsenate almost completely prevents the cells from being killed by the bacteriocin when either glucose or L-lactate is applied. Concomittantly, the ATP-level is decreased under both conditions. However, L-glutamic acid uptake is only impaired in glucose-stimulated cells. These results suggest that phosphate-bond energy is required in rendering cells sensitive to staphylococcin 1580. This conclusion is strengthened by the observations made with DCCD, an inhibitor of the (Mg^{2+}, Ca^{2+})-ATPase. Glucose-stimulated cells, which can derive ATP from substrate phosphorylation, are equally sensitive both in the presence and absence of DCCD. Cells incubated with L-lactate, which presumably derive ATP only from the protonmotive force via the (Mg^{2+}, Ca^{2+})-ATPase, are less sensitive to staphylococcin in the presence of the inhibitor. The partial inhibition of L-glutamic acid uptake in the experiments with DCCD may reflect the involvement of the (Mg^{2+}, Ca^{2+})-ATPase in the active transport, as was shown in *Bacillus subtilis* (1) and *E. coli* (23).

Uncouplers, such as FCCP and valinomycin in the presence of K^+, prevent reenergization both with glucose and L-lactate. In this respect it should be noted that uncouplers do not block the ac-

TABLE 4. THE EFFECT OF ANAEROBIOSIS ON THE KILLING EFFECT OF STAPHYLOCOCCIN 1580

Growth conditions[a]	Incubation conditions[a] and additions		log R_t TSA	log R_t TSAS	ATP-concentration (nmol/mg dry wt)
aer., starved[b]	aer.	none	0.0	-0.20	0.1
	aer.	glucose	-1.05	-2.70	6.4
	an.	glucose	-0.94	-2.35	4.8
	an.+DT	glucose	-0.70	-1.92	3.8
aer.	aer.	none	-0.82	-3.10	-
an.	an.	none	-0.40	-1.00	-
		glucose	-0.60	-2.28	-
an.+$NaNO_3$	an.	none	-0.62	-1.62	-
	an.	glucose	-0.79	-2.67	-
	an.	L-lactate	-0.56	-1.60	-
	an.+$NaNO_3$,	L-lactate	-0.75	-2.40	-

S. aureus cells were grown either aerobically or anaerobically with glucose as described. Cell suspensions, containing about 6×10^8 cells per ml, were incubated aerobically or anaerobically with N_2 bubbling through the suspension, either in the presence or absence of sodium dithionite (DT, 1 mM). Where indicated, glucose (20 mM), L-lactate (10 mM) and sodium nitrate (20 mM) were added and incubation took place for 5 min at 37°C. Then, samples were removed to measure the ATP level and the remainder received staphylococcin 1580 (100 arbitrary units per ml). 5 min later the survival ratio was measured by plating on both TSA and TSAS.
- not tested
[a] aer. = aerobically, an. = anaerobically
[b] starved after growth

tion of staphylococcin 1580 in normal cells, when added only shortly prior to the addition af the bacteriocin (A. Weerkamp, unpublished observation), which also suggests that the protonmotive force is not directly involved in the initiation of the staphylococcin 1580 action. Cyanide exerted a stronger effect, both on the initiation of the action of staphylococcin 1580 and

on the energized state of glucose-stimulated cells, than would be expected from inhibition of the respiration only. However, it will be shown hereafter that the respiratory chain is not directly involved in the initiation of the action of the bacteriocin.

The effect of anaerobiosis

Starved, aerobically grown cells, are slightly less sensitive to staphylococcin 1580 under anaerobic incubation conditions in the presence of glucose (Table 4). Dithionite further decreases the sensitivity, but the cells are still substantially killed by the bacteriocin. Anaerobic incubation gives rise to a decrease in ATP level, about proportionally to the decrease in sensitivity. Cells, anaerobically grown with glucose, either in the presence or absence of nitrate, are relatively insensitive to staphylococcin 1580 when they are incubated without an exogenously added energy source (Table 4). Glucose restores sensitivity to values comparable to those obtained with aerobically grown cells. In addition, L-lactate stimulates the sensitivity to staphylococcin 1580 in cells previously induced for the synthesis of nitrate reductase (3), in the presence, but not in the absence of nitrate.

Effect of staphylococcin 1580 on mutants defective in energy metabolism

The energy requirement for the initiation of the action of colicins has been elegantly studied by the use of a mutant of *E. coli* defective in the (Mg^{2+}, Ca^{2+})-ATPase (14). Unfortunately, such mutants have not been described for other bacterial species, except for *B. megaterium* (9). Numerous attemps to obtain true ATPase$^-$ mutants of *S. aureus* by various techniques applied to *E. coli* failed in our hands.

Results obtained with some mutants, otherwise defective in the transformation of energy, are shown in Table 5. Strain SA4 fails to grow on non-fermentable substrates, but is not impaired in respiration, L-lactate oxidation and (Mg^{2+}, Ca^{2+})-ATPase activity. Active uptake of amino acids is strongly reduced both in test where glucose was used as carbon source as well as when

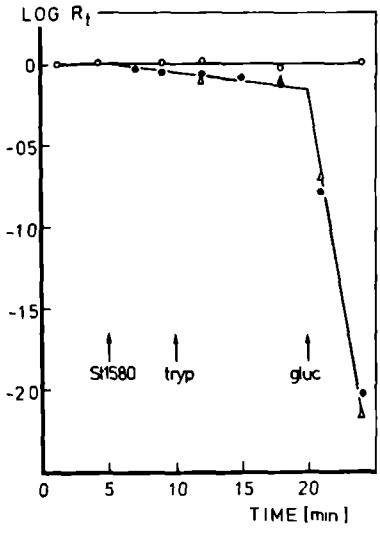

Fig. 3. Effect of incubation with trypsin on the action of staphylococcin 1580 in starved cells of *S. aureus*. Starved cells (6×10^8 cells per ml), prepared as described in Fig. 1, were incubated at 37°C with staphylococcin 1580 (100 arbitrary units per ml) or only with buffer. After 5 min the suspensions were split into two portions, one of which received trypsin (0.5 mg per ml), and further incubated, followed 10 min later by the addition of glucose (20 mM) to each of the incubations. At various times samples were removed and the viability was measured by plating on TSAS.
O, received all additions except staphylococcin 1580;
Δ, received all additions except trypsin; ●, received all additions.

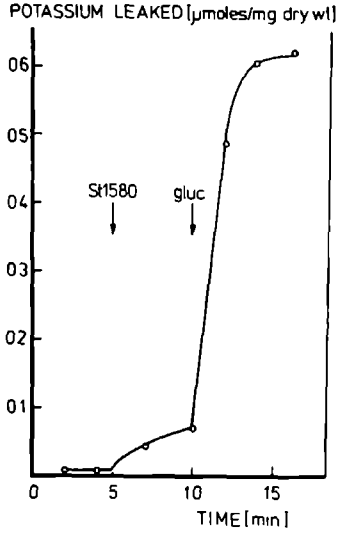

Fig. 4. Effect of reenergization of starved cells on the induction of leak of potassium by staphylococcin 1580.
A suspension of starved cells (1×10^9 cells per ml) was prepared as described in Fig. 1, except that the cells were washed and finally resuspended in 50 mM Tris·HCl (pH 7.0), 50 mM sodium chloride and 10 mM magnesium sulfate, and incubated at 37°C without an exogenous carbon source. After 5 min staphylococcin 1580 (150 arbitrary units per ml) was added, followed 5 min later by glucose (20 mM). At various times samples were taken, the cells removed by centrifugation for 2 min at 18.000 x *g*, and the potassium concentration of the supernatant was measured.

lactate was used. Hence it follows that in this mutant the maintenance of a high-energy state of the membrane is impaired. In agreement with the results obtained with the parent strain, glucose is able to induce staphylococcin sensitivity, but L-lactate completely fails to do such. This supports the previous conclusion that phosphate-bond energy is involved in the initiation of staphylococcin 1580-sensitivity.
Strain M17, which is auxotrophic for menadione and lacks a functional respiratory chain when grown in the absence of menadione (10), is only slightly attacked by staphylococcin 1580, presumably due to the low internal ATP level. Addition of glucose, but not of L-lactate, improves the killing effect of the bacteriocin. Hence, it is concluded that the respiratory chain is not directly involved in the initiation of the action of staphylococcin 1580.

The penetration of the cell wall

Staphylococcin 1580 is sensitive to trypsin, but cells incubated with the bacteriocin cannot be rescued by trypsin-treatment (13). Similarly, starved cells are not rescued by trypsin in the period before the energization by the exogenous carbon source (Fig. 3), since killing proceeds in an unaltered way after glucose addition. This suggests that staphylococcin 1580 penetrates the cell wall region also in the absence of metabolic energy, or that once adsorbed bacteriocin is no longer susceptible to trypsin.

Effect of staphylococcin 1580 on potassium leak

Since the parameters used to measure the effect of staphylococcin 1580 so far, such as viability and amino acid uptake, are themselves energy dependent, the effect of the bacteriocin on the energy-independent leak of potassium ions from the cells was investigated. Fig. 4 shows that starved cells do not become leaky when incubated with staphylococcin 1580, until glucose is added to the incubation mixture. This suggests that the membrane is resistant to the action of staphylococcin 1580 in the absence of substrates which provide metabolic energy.

TABLE 5. EFFECT OF STAPHYLOCOCCIN 1580 ON *S. AUREUS* MUTANTS DEFECTIVE IN THE ENERGY METABOLISM

Strain	Growth conditions	Carbon source	log survival ratio at 5 min: cells plated on TSA	log survival ratio at 5 min: cells plated on TSAS	ATP-concentration (nmol/mg dry wt)	L-glutamic acid uptake (nmol/min per mg dry wt)
Oxford 209P	A-glucose, starved	none	-0.01	-0.02	0.6	0.02
		glucose 20mM	-0.85	-2.60	6.7	0.12
		L-lactate 10mM	-0.08	-0.92	9.2	0.40
SA4	A-glucose, starved	none	-0.01	-0.05	0.4	0.01
		glucose 20mM	-0.36	-2.48	5.9	0.03
		L-lactate 10mM	-0.02	+0.06	2.3	0.05
M17	AN-glucose +menadione, not starved	none	-1.10	-2.50	2.2	-
M17	AN-glucose -menadione, not starved	none	-0.10	-0.22	0.7	-
		glucose 20mM	-0.45	-1.90	2.8	-
		L-lactate 10mM	-0.10	-0.30	0.8	-

Cells were grown into mid-exponential phase under the conditions indicated, and where indicated, the cells were starved as described in Materials and Methods.
The experiments were carried out as described in the legends of Table 1.

- not tested

The cell membrane, which is presumed to be the target of the action of staphylococcin 1580 (12,14,30) is accessible to the bacteriocin, even in the absence of substrates which yield metabolic energy. However, this access is not accompanied with the induction of physiological damage. An effective adsorption to the reactive site of the membrane does not take place in the absence of a carbon source and the adsorption appears completely reversible when the cells are diluted into bacteriocin-free medium, as done in the plating experiments. In this, staphylococcin 1580 differs from the colicins, since the latter require the addition of trypsin (24) or sodium dodecyl sulfate (4) to rescue the cells from irreversible damage. Trypsine rescue has also been demonstrated with bacteriocins attacking gram-positive bacteria such as staphylococcin C55 (7) and enterocin E1-A (17), but was ineffective in cells once treated with staphylococcin 1580. Upon reenergization, sites sensitive to staphylococcin 1580 might become exposed to the outside of the membrane as a result of a conformational change within the membrane, allowing the attack by the bacteriocin. Alternatively, energy may be required to transmit the bacteriocin, or part of it, through the cell membrane, or to spread the primary effect of the bacteriocin by means of energy-dependent conformational changes (5,19, 22). Such hypotheses have also been proposed for the initiation of the action of colicins (14). The first hypothesis may be supported by the observation that energization of the membrane increased the accessibility of the lac carrier protein in *Escherichia coli* (26).

Several lines of evidence, particularly the effects of arsenate and DCCD and the results obtained with mutant strain SA4, suggest that phosphate-bond energy is required to initiate sensitivity to staphylococcin 1580 in starved, glucose-grown cells. In contrast, the initiation of colicin action apparently requires a high energy state of the membrane (14). However, it is apparent that ATP itself is not directly involved as an activator in the staphylococcin 1580 action, since L-lactate stimulates sensitivity only weakly, in spite of the induction of high

intracellular ATP-levels. A plausible explanation might be that an intermediate with a high-energy phosphate bond is involved in a mechanism regulating the energy transduction, presumably at the level of the cell membrane. The observed increase in the NADH level with glucose, as compared to L-lactate, may be another reflection of this regulation mechanism, or NADH may be directly involved in the regulation, considering its central place in energy-transformation. The results obtained with cells grown with pyruvate as carbon source cannot be fully clarified. Presumably, enough endogenous energy remained in these cells after starvation to enable the initiation of action of staphylococcin 1580. It was previously noted (30) that unstarved, pyruvate-grown cells are more sensitive to the bacteriocin than cells grown with glucose.

Some experimental data may support the hypothesis outlined above. It is known that intracellular sugar phosphates regulate the uptake of nutrients by different uptake systems in *S. aureus* (27). In *E. coli*, mutants tolerant to colicin K (25) and I (16) have been found pleiotropically affected in several parts of the aerobic metabolism. In addition, a temperature-sensitive mutant (ecf^{ts}) has recently been described by Lieberman and Hong (18) that mimics at the nonpermissive temperature cells treated with colicin E1 or K. It was suggested that this mutant was defective in a protein which is involved in the regulation of energy transduction and is simultaneously the target for colicins E1 and K.

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CHAPTER 8

EFFECTS OF STAPHYLOCOCCIN 1580 ON CELLS AND MEMBRANE VESICLES OF *BACILLUS SUBTILIS* W23

INTRODUCTION

Bacteriocins are protein antibiotics, which are bactericidal to bacteria related to the producing strain. One class of bacteriocins is assumed to exert its action directly on the cytoplasmic membrane. Among them, especially colicins E1 and K (23) have been studied extensively. Also staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis*, has been studied to considerable depth (12,13,29,30). Like colicins E1 and K, staphylococcin 1580 rapidly induces an inhibition of macromolecular synthesis and active transport, depletion of cellular ATP and leak of preaccumulated Rb^+ from the cells (12). However, the permeability to protons is not affected (13,30).
Sensitivity of cells to staphylococcin 1580 is dependent on the physiological state of the cells, and is influenced by conditions such as the temperature during growth and incubation, energy charge of the cells and the kind of carbon source applied in the medium (30). It was suggested that the primary effect of staphylococcin 1580 is the uncoupling of the membrane potential and electron transport (30).
Jetten and Vogels (13) showed that, in accordance to results obtained with colicins E1 (1) and A (13), transport of amino acids in membrane vesicles was inhibited by staphylococcin 1580. However, relatively large amounts of staphylococcin 1580 were required to exert this effect. This study aims to compare the action of staphylococcin 1580 on whole cells and membrane vesicles under comparable conditions, and to extend the previous experiments as to the mode of action of the bacteriocin.
In the present study *Bacillus subtilis* W23, an organism equally sensitive to staphylococcin 1580 as the previously used *Staphylococcus aureus* (13) was used as the indicator strain. Uptake of various substrates into vesicles prepared from *B. subtilis* W23 has been extensively studied by Konings *et al*

(3,18,24). They showed that these vesicles are essentially right side out and that transport rates of vesicles approximate those of intact cells.

MATERIALS AND METHODS

Bacterial strain, medium and growth conditions

B. subtilis W23 was grown in nutrient-sporulation medium (8) at 37°C with vigorous aeration. The medium was inoculated with an overnight grown culture to obtain an absorbancy at 600 nm (A_{600}) of 0.05, and harvested in mid-exponential phase (A_{600} 1.2). The cells were washed once with 0.1M potassium phosphate (pH 7.3), and centrifuged 10 min at 12,000 x *g*.

Preparation of membrane vesicles

Membrane vesicles were prepared by direct lysis in hypotonic medium, as described by Konings and coworkers (3,19), with the exception that the cells were harvested in the mid-exponential rather than in the early stationary phase. Sodium-loaded vesicles were prepared by an identical procedure, except that sodium buffers replaced potassium buffers throughout the procedure. Vesicles were rapidly frozen in thin-walled plastic tubes by the use of liquid nitrogen and stored at -80°C. Immediately before use vesicles were rapidly thawed at 46°C in a waterbath; only vesicles that were frozen once were used.

Transport experiments

Incubation mixtures contained, unless otherwise indicated, final concentrations of either 50 mM potassium or sodium phosphate (pH 6.6), 10 mM magnesium sulfate and 0.2 - 0.5 mg of membrane protein per ml in a total volume of 50 - 250 µl. Mixtures were incubated at 25°C with a stream of water-saturated oxygen blowing over the surface. After 2 min preincubation the electron donor was added, followed one min later by the labelled substrate. Initial uptake rates were measured after 30 sec by terminating the reaction by the addition of 2 ml 0.1M LiCl, as described earlier (13). The time course of uptake was measured by removing samples from the incubation mixture at vari-

ous time intervals with an Oxford sampler (Oxford Lab. Intern. Corp.). Samples were spotted on a prewetted 0.45 μ pore size membrane filter (Millipore Corp.) and washed with 3 ml 0.1M LiCl, at room temperature.
Uptake experiments in whole cells (about 1.2 mg dry weight per ml) were performed as described for membrane vesicles.

Fluorescence measurements
Fluorescence of 3,3'-dipropylthiodicarbocyanine (DiS-C_3) was used to monitor the membrane potential (2,27) and was carried out as described previously (30). The incubation mixtures (2.5 ml final volume) contained 50mM of either sodium or potassium phosphate (pH 6.6), 10mM $MgSO_4$ and 20 μg membrane protein per ml. DiS-C_3 was added to a concentration of 0.8 μM and fluorescence was excited at 620 nm and measured at 662 nm, in an Aminco Bowman spectrofluorimeter. Measurements were carried out at 25°C. The percent fluorescence quenched was calibrated with a potassium diffusion potential to estimate the respiration-induced membrane potential. Potassium-loaded vesicles were diluted into phosphate buffers containing different proportions of sodium and potassium phosphate. The fluorescence quench occurring upon addition of valinomycin was recorded and plotted against the diffusion potential, calculated from the Nernst equation ($\Delta\psi = 59 \log K^+$ in/K^+ out).
Our results were similar to those obtained by Bhattacharryya *et al* (2) who used *Azotobacter vinelandii* vesicles.

Preparation and assay of staphylococcin 1580
Staphylococcin 1580 was prepared from supernatants of overnight-grown cultures of *S. epidermidis* 1580 on a semisynthetic medium as described earlier (29). The activity was assayed with *S. aureus* Oxford 209P as indicator strain (10).

Determination of viability
Cells were incubated at 25°C as described for the uptake experiments. After 2 min of preincubation, staphylococcin 1580 was added and after another 4 min samples were removed, rapidly diluted 1000-fold in peptone water (Oxoid) and plated in suita-

ble dilutions on nutrient agar (Oxoid) containing 0.2M NaCl. The results were expressed as percent survivors relative to cell suspensions not treated with staphylococcin 1580.

Protein determination

Protein was measured according to the method of Lowry *et al* (22). The membrane protein content of whole cells was assumed to be 15% of the total protein content (15).

Chemicals

3,3'-dipropylthiodicarbocyanine (DiS-C_3) was a gift from A. Waggoner, Amherst College, Amherst Mass. Nigericin was donated by Dr. W.E. Scott, Hoffman La Roche, Nutley, N.J. Valinomycin was obtained from Calbiochem, Lucerne, Switzerland; gramicidin D was from Boehringer, Mannheim, W-Germany, and 5-N-methyl phenazonium methyl-sulfate (PMS) from BDH-Chemicals Ltd, Poole, England.

Radiochemicals, obtained from Radiochemical Centre, Amersham, England, were L-[U-^{14}C]glutamic acid (270mCi/mmol), L-[U-^{14}C] glutamine (40mCi/mmol), L-[U-^{14}C]leucine (330mCi/mmol), L-[U-^{14}C]lactate sodium salt (50mCi/mmol), [1-^{14}C]acetate sodium salt (58mCi/mmol), [^{3}H]inuline (695mCi/mmol), ^{86}RbCl (2-10mCi/mg), and methyl (α-D-[U-^{14}C]gluco)pyranoside (3mCi/mmol).

RESULTS

Effect of staphylococcin 1580 on active transport in whole cells and membrane vesicles of B. subtilis

It was previously shown that staphylococcin 1580 blocks active uptake of amino acids in cells (12) and membrane vesicles (13) of *S. aureus*, and causes the efflux of preaccumulated solute. Fig. 1 shows that uptake of L-glutamate in cells of *B. subtilis* is inhibited and that the preaccumulated amino acid is rapidly released under the influence of the bacteriocin. However, similar concentrations of staphylococcin 1580 have much less severe effect on the uptake of α-methylglucoside. Relatively low concentrations of staphylococcin 1580 even enhance uptake significantly, up to a maximal stimulation of 40% under the condi-

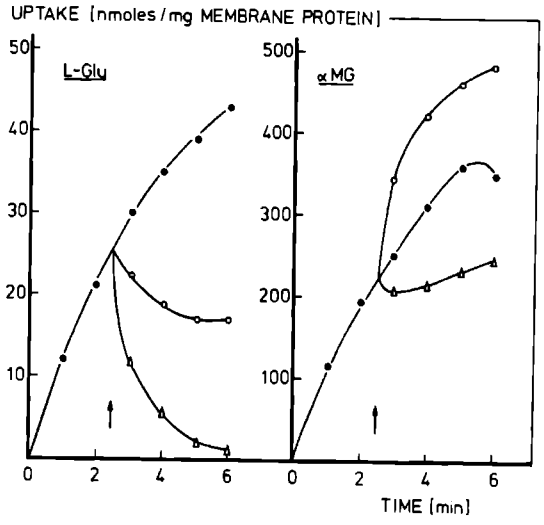

Fig. 1.
Effect of staphylococcin 1580 on the uptake of L-glutamate (L-Glu) and α-methylglucoside (αMG) by cells of *B. subtilis* W23. Cells were grown into mid-exponential phase, washed and resuspended in 50mM potassium phosphate (pH 6.6), and 10mM magnesium sulfate, at a final concentration of 1.2 mg dry weight per ml. Uptake experiments were carried out at 25°C as described, but an exogenous energy source was omitted. Staphylococcin 1580 was added 2.5 min (indicated by the arrow) after the addition of the label in a final concentration of 10 (○) and 350 arbitrary units per ml (△), respectively. Controls received no staphylococcin (●). The uptake of L-glutamate (11.7 µM) and α-methylglucoside (1.5mM) was plotted against the time.

tions tested. In *B. subtilis* α-methylglucoside is transported by the phospho*enol*pyruvate : glucose phosphotransferase system, and retained within the cell both as α-methylglucoside phosphate and, after dephosphorylation, as α-methylglucoside, which are not further metabolized under the experimental conditions (6). The retention of α-methylglucoside within the cells indicates that the integrity of the cell membrane is conserved when staphylococcin was applied in concentrations that effectively inhibited glutamate uptake. The uptake of α-methylglucoside was

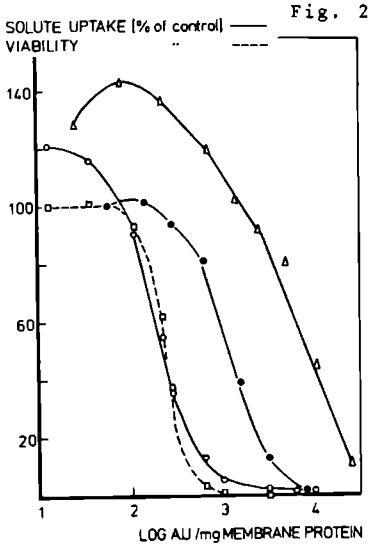

Fig. 2. Correlation between the concentration of staphylococcin 1580 and its effect on the viability of cells, on L-glutamate uptake in cells and membrane vesicles and on α-methylglucoside uptake in cells. Uptake rates of L-glutamate (11.7 µM) and α-methylglucoside (1.5mM) were measured after 30 sec, and amounted to 12.2 and 115.0 nmol/min per mg membrane protein, respectively, when cells were tested, whereas 9.4 nmol L-glutamate were taken up per min and per mg membrane protein in tests with vesicles. Staphylococcin 1580, dissolved in buffer, was added one min prior to the addition of the labelled compound; controls received only the buffer. Uptake in membrane vesicles was stimulated by sodium ascorbate (20mM) and PMS (0.1mM); in experiments with whole cells no exogenous energy source was added. The viability was measured after treatment of the cells with staphylococcin for 4 min. Results were expressed as the percentage of control experiments without staphylococcin 1580, and plotted as a function of the staphylococcin concentration.
Open symbols: whole cells, L-glutamate uptake (O); α-methylglucoside uptake (Δ); viability (□).
Closed symbols: membrane vesicles, L-glutamate uptake (●).

inhibited when higher concentrations of the bacteriocin were used (Fig.2). This effect is presumably due to an increase of the permeability of the membrane. Similar results have been reported for *Escherichia coli* cells treated with colicins E1 and K (14) or Ia (9), except that several fold stimulations were found with cells grown on glycerol.

The killing of the cells correlates with the inhibition of L-glutamate uptake (Fig. 2) if the bacteriocin concentration is expressed as arbitrary units per mg membrane protein. This way of plotting was chosen in order to compare the effect of staphylococcin 1580 on both cells and membrane vesicles and is based on the assumption that the membrane is the direct target for the action of staphylococcin 1580. If expressed in this way

TABLE 1. EFFECT OF STAPHYLOCOCCIN 1580 ON THE UPTAKE OF VARIOUS SUBSTRATES BY MEMBRANE VESICLES OF *B. SUBTILIS* W23, ENERGIZED BY VARIOUS ELECTRON DONORS

Substrate	electron donor system	Uptake (nmol/min per mg protein		% Inhibition
		-st1580	+st1580	
L-glutamate	ascorbate + PMS	12.80	11.20	12.6
L-glutamate	succinate + PMS	4.20	3.59	14.5
L-glutamate	NADH	10.48	8.59	10.5
L-leucine	NADH	1.00	0.78	22.0
L-glutamine	NADH	1.33	0.57	57.2
L-lactate	NADH	4.30	1.53	64.5
acetate	NADH	0.21	0.15	28.5
Rb^+ (in the presence of valinomycin)	NADH	30.4	24.0	21.0

Potassium-loaded vesicles (0.37 mg protein per ml) were incubated at 25°C with 50mM potassium phosphate (pH 6.6), and 10mM magnesium sulfate in a final volume of 55 µl. 250 arbitrary units of staphylococcin 1580 (675 arbitrary units per mg membrane protein) were added immediately prior to the addition of the electron donors. After 1 min of incubation the uptake was started by the addition of the labelled compounds. The uptake was terminated 30 sec later and the radioactivity was measured. The electron donors used were: NADH (5mM), sodium ascorbate (20mM) and PMS (0.1mM); sodium succinate (10mM) and PMS (0.2mM). Final concentrations of the substrates: L-glutamate, 20.0 µM; L-leucine, 21.7 µM; L-glutamine, 83.3 µM; L-lactate, 133.0 µM; acetate, 78.0 µM; and RbCl, 2mM.
The uptake of acetate and Rb^+ was measured as steady state levels (nmol/mg protein) instead of initial rates, after 6 and 2 min respectively. Blanks obtained in the presence of nigericin (0.5 µm), which completely abolishes the pH gradient over the membrane (25), were subtracted from the values obtained with acetate. Moreover, these values represent only minimum ones, since the label is rapidly lost from the vesicles during the wash on the filter (25). To measure the uptake of Rb^+, sodium-loaded vesicles (0.21 mg protein per ml) and sodium phosphate buffers were applied, and 150 arbitrary units of staphylococcin 1580 per ml (710 arbitrary units per mg membrane protein) were used. The uptake was expressed relative to values obtained in the absence of valinomycin.

transport rates for L-glutamate were about similar in whole cells and membrane vesicles (19,20), but a 5 times higher staphylococcin 1580 concentration is required to inhibit transport in membrane vesicles to the same extent as in whole cells. This difference presumably is not the result of a difference in the amount of staphylococcin 1580 adsorbed by either cells or vesicles, since whole cells can adsorb more bacteriocin than vesicles can (under the experimental conditions about 750 and 250 arbitrary units per mg membrane protein, respectively). It was previously shown that staphylococcin 1580 has a strong tendency to stick aspecifically to both cell walls and membranes of sensitive as well as resistant bacteria (11). It should be noted, however, that the ratio between specific and unspecific adsorption is unknown.

Table 1 shows the effect of staphylococcin 1580 on the uptake of various compounds by membrane vesicles, and the influence of various electron donor systems. The initial uptake of L-glutamate was inhibited to the same extent, whenever NADH, succinate-PMS, or ascorbate-PMS were used as electron donors. This result is conceivable, since staphylococcin 1580 does not affect the electron transport, or membrane-bound dehydrogenases (13). The differences observed between the transport inhibition of various substrates by the bacteriocin cannot simply be traced down to differential effects of staphylococcin 1580 on the membrane pH-gradient (uptake of acetate) and on the membrane potential (uptake of rubidium in the presence of valinomycin). This suggests that staphylococcin 1580 has a differential effect on either the functioning of the various transport carriers or their coupling to the high-energy state of the membrane, rather than on the transport driving force.

Effect of staphylococcin 1580 on anaerobic influx

Fig. 3 shows that under anaerobic conditions, which strongly inhibited the active accumulation of L-glutamate and the oxidation of NADH (4), externally added L-glutamate does not completely equilibrate with the intramembranal pool in the presence of staphylococcin 1580. An alternative interpretation of the data might be that the label is rapidly lost from staphylococ-

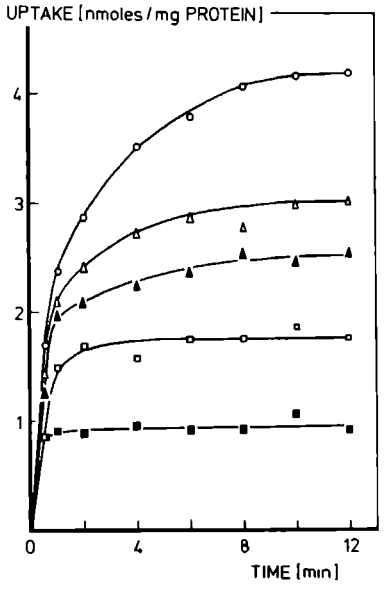

Fig. 3. Effect of staphylococcin 1580 on the influx of L-glutamate in membrane vesicles under anaerobic conditions. Membrane vesicles (1.88 mg protein per ml) were incubated at 25°C in a final volume of 225 µl under a stream of nitrogen. After 2 min NADH (final concentration 5mM) was added, and the mixture was incubated anaerobically for 5 min more. Then staphylococcin 1580 was added, followed 3 min later by the labelled L-glutamate. Samples (25 µl) were removed at various time intervals, washed with 2 ml 0.1M LiCl and the radioactivity was determined. The final concentration of L-glutamate was 2mM (4.9mCi/mmol).
Symbols: 0 (0); 8 (△); 34 (▲); 85 (□); 337 (■) arbitrary units of staphylococcin 1580 per ml.

cin-treated vesicles during the washing procedure. However, two observations favoured the first given conclusion: (i) similar experiments were carried out without this washing and the amount of extravesicular water remaining on the filters was monitored with [^{3}H] inulin, which is presumably excluded by the vesicles. The results were somewhat variable, but resemble those shown in Fig 3., (ii) the rate of efflux of label during the washing procedure was similar for staphylococcin-treated and untreated vesicles (result not shown).
The bacteriocin-concentrations required to induce a distinct effect on the glutamate influx are much lower than those required to inhibit active glutamate uptake, which is particularly clear when the relatively high membrane concentrations used in the anaerobic experiments are taken into account.

Effect of staphylococcin 1580 on the L-glutamate carrier
The effect of staphylococcin 1580 on the efflux of preaccumulated L-glutamate from membrane vesicles, was studied by diluting

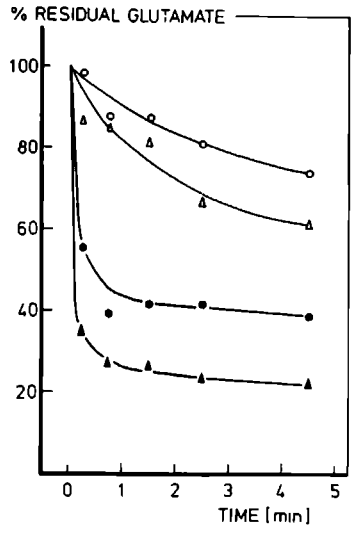

Fig. 4. Effect of staphylococcin 1580 on the L-glutamate carrier system under efflux conditions. Membrane vesicles (0.35 mg protein per ml) were preloaded for 8 min with ^{14}C-labelled L-glutamate (20.0 µM) in the presence of 5mM NADH. Final volume of the incubation mixture was 110µl. Subsequently, staphylococcin 1580 (250 arbitrary units per ml) or only buffer were added and two min later the incubation mixtures were rapidly diluted 50-fold with prewarmed incubation buffer, containing either 5mM or no NADH, and [^{3}H] inuline (0.5µCi/ml). Samples (1 ml) were taken at various time intervals and filtered without further washing. The amount of extravesicular water remaining on the filter was calculated from the ^{3}H radioactivity. The results are expressed as percentage L-glutamate remaining in staphylococcin-treated or untreated vesicles after dilution, with regard to the L-glutamate concentration in vesicles that were not diluted.
Symbols: Controls diluted in medium containing 5mM NADH (○); staphylococcin 1580-treated, diluted in medium containing 5mM NADH (△); controls, diluted in medium without NADH (resulting in a final concentration of 0.1mM) (●); staphylococcin 1580-treated, diluted in medium without NADH (final concentration 0.1mM (▲).

bacteriocin-treated and untreated vesicles 50-fold, without interfering with the energy supply (Fig.4). The rate of glutamate efflux is distinctly higher in the staphylococcin-treated vesicles, and apparently the newly reached steady state level is lower than in control vesicles. When the NADH-concentration was simultaneously diluted 50-fold, and thus the NADH oxidation rate was lowered, comparable results were obtained.

Fig. 5 shows the effect of staphylococcin 1580 on the exchange properties of the vesicles. Preaccumulated ^{14}C-labelled L-glutamate exchanged slightly more rapidly with exogenously added unlabelled L-glutamate when the vesicles were treated first with rather high amounts of staphylococcin 1580.

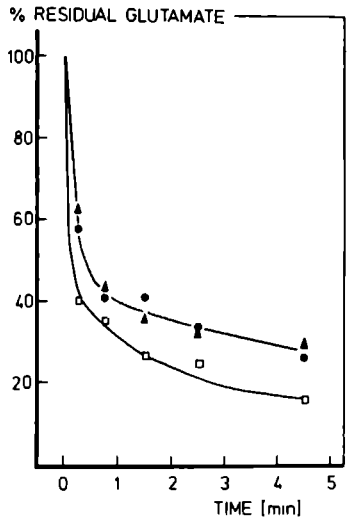

Fig.5. Effect of staphylococcin 1580 on L-glutamate exchange. The conditions were the same as in Fig. 4, except that the vesicles were diluted 50-fold in a buffer medium containing 5mM NADH and 2mM unlabelled L-glutamate. Symbols: vesicles not treated with staphylococcin 1580 (●); treated with 100 arbitrary units per ml (▲); treated with 250 arbitrary units per ml (□).

These results suggest that staphylococcin 1580 may have some direct effect on the L-glutamate carrier functions. However, the observed effects may also be an indirect result of the interference of staphylococcin 1580 with the energy supply.

Effect of staphylococcin 1580 on DiS-C_3 fluorescence

Recently, Bhattacharryya *et al* (2) used the fluorescent dye DiS-C_3 to monitor the membrane potential in membrane vesicles of *Azotobacter vinelandii*. The fluorescence quench can be directly correlated to the membrane potential (in mV) by calibration with the diffusion potential posed on potassium-loaded vesicles upon addition of valinomycin. Our results (not shown) obtained with membrane vesicles of *B. subtilis* were similar to those reported by Bhattacharryya *et al* (2). Fig. 6 shows that vesicles quenched the DiS-C_3 fluorescence upon addition of NADH (steady state 99mV). Addition of staphylococcin 1580 instantaneously abolished the fluorescence quench to a final level which depends on the amount of bacteriocin applied; the result-

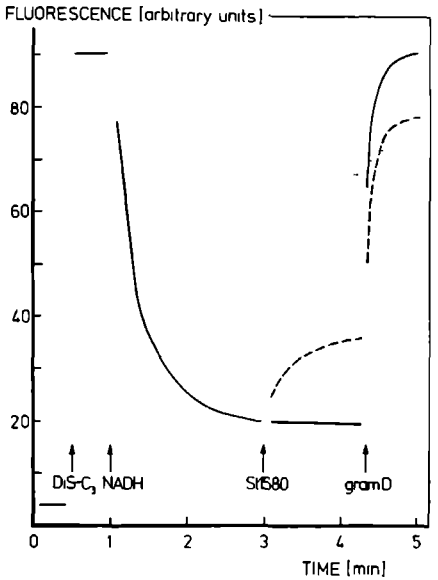

Fig. 6. Effect of staphylococcin 1580 on the respiration induced quenching of DiS-C_3 fluorescence. Membrane vesicles (20 µg protein/ml) were incubated at 25°C in 50mM potassium phosphate (pH 6.6), 10mM magnesium sulfate and 0.8µM DiS-C_3. Respiration was started by the addition of 5mM NADH. When the fluorescence quench reached a plateau, staphylococcin 1580 in a final concentration of 2500 arbitrary units per mg membrane protein (dotted line), or 500 arbitrary units per mg membrane protein (broken line), or only buffer (solid line) was added. At the end of the experiment gramicidin D (1µg/ml) was added, which completely abolishes the membrane potential.

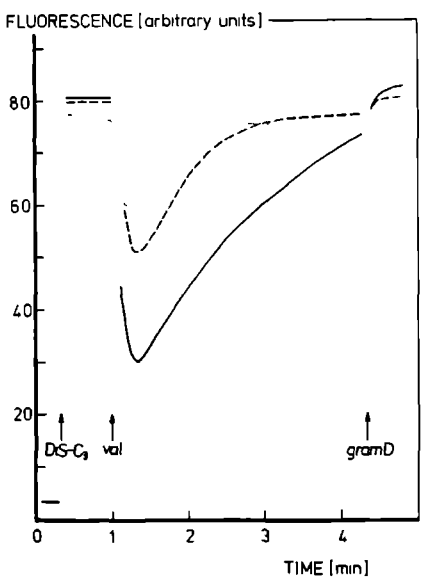

Fig. 7. Effect of staphylococcin 1580 on the DiS-C_3 fluorescence quenching, induced by a potassium-diffusion potential. Potassium-loaded membrane vesicles (20µg protein per ml) were incubated for 2 min at 25°C in a buffer containing 49mM sodium phosphate and 1mM potassium phosphate (pH 6.6), 10mM magnesium sulfate, and staphylococcin 1580 in a final concentration of 2500 (dotted line) and 500 arbitrary units per mg membrane protein (broken line) or buffer instead of the bacteriocin (solid line). Then DiS-C_3 (0.8µM) was added, followed about 1 min later by valinomycin (0.5µM). At the end of the experiment gramicidin D (1 µg/ml) was added.

ing membrane potential was 79 mV and 40 mV after treatment with 500 and 2500 arbitrary units of staphylococcin 1580 per mg membrane protein, respectively.
Staphylococcin 1580 abolishes also the membrane potential built up by the diffusion potential of potassium ions (Fig. 7). In the presence of the bacteriocin a lower optimum was reached and the quench was dissipated more rapidly,
Almost no influence of staphylococcin 1580 was observed on the DiS-C_3 fluorescence with "resting" vesicles, irrespective of sodium- or potassium-loaded vesicles were used in the presence of either sodium- or potassium phosphate buffer. Therefore staphylococcin 1580 cannot be regarded as a specific conductor of either potassium or sodium ions.

DISCUSSION

Many observations have pointed to the cytoplasmic membrane as the direct target for the action of staphylococcin 1580 (12,13, 30). Therefore, a number of effects of the bacteriocin on isolated membrane vesicles of *B. subtilis* were studied and compared to the effects on whole cells. However, a direct comparison between the results obtained with vesicles and cells is impeded by structural and functional differences of both. Konings (19, 20) has shown that the membrane of *B. subtilis* W23 vesicles, prepared by the method used in this study, are topologically identical to the membranes of intact cells. Moreover, the specific transport rates of these vesicles approximate those of intact cells when expressed as moles transported per weight unit of membrane protein. However, vesicles lack staphylococcin 1580-reactive sites (receptors) which may be involved in whole cells to foster the bacteriocin to its biochemical target, and are also devoid of cell wall-type aspecific binding sites for the bacteriocin. Moreover, the complex balance between intra- and extracellular solutes in whole cells may result in differences in the sensitivity to the bacteriocin on comparing whole cells with membrane vesicles. This may be illustrated by the suggestion of Brewer (5) that after colicin K-treatment the membrane potential of *E. coli* cells depends on an electrochemi-

TABLE 2. CLASSIFICATION OF EFFECTS INDUCED BY STAPHYLOCOCCIN 1580 IN *B. SUBTILIS* CELLS AND MEMBRANE VESICLES

System	Process	Amount of bacteriocin yielding a half-maximal effect (arbitrary units/ mg membrane protein)
vesicles	Anaerobic L-glutamate influx, steady state level	20 - 60
vesicles	Active uptake of solutes coupled to the high-energy state of the membrane[a]; membrane potential (fluor.) L-glutamate efflux	400 -1600
cells	L-glutamate uptake; killing	250
cells	α-methylglucoside uptake	9400

[a] solutes listed in Table 1.

cal potential of an anion. In accordance, anions, such as pyruvate and phosphorylated intermediates of glycolysis, are lost after colicin E1 or K treatment (7).

The results obtained in this investigation allow a classification of the effects of staphylococcin 1580 into at least three groups (Table 2). The anaerobic influx of L-glutamate in membrane vesicles is most sensitive to the bacteriocin. At present, the way in which staphylococcin 1580 affects this influx is not clear. The kinetics of solute permeation has been extensively studied with membrane vesicles of *E. coli* (16,21) and *S. aureus* (26). In *S. aureus* vesicles serine efflux, and influx under energized conditions, exhibited saturation kinetics. However, anaerobic influx appeared to be a nonsaturable process, and it was concluded that the transport carriers catalyze facilitated diffusion in the direction of efflux only (26). If this conclusion may be extended to glutamate influx in *B. subtilis* vesicles, staphylococcin 1580 would specifically block this passive diffusion, possibly as a result of an effect on the membrane

charge or the hydrophobicity of the diffusion barrier.
A second group of effects are exerted by staphylococcin 1580 when applied to cells or membrane vesicles in concentrations of about 250 and 400 - 1600 arbitrary units per mg of membrane protein, respectively. These effects can be explained by a dissipation of the proton motive force, since $\Delta\psi$ (rubidium uptake in the presence of valinomycin; DiS-C_3 fluorescence) and ΔpH (acetate uptake) of membrane vesicles are abolished by these bacteriocin concentrations. In this view the differences between the inhibitory effects on individual transport systems, observed in membrane vesicles, may be the result of a different sensing of the transport carriers to the energy supply. Alternatively, an alteration of the membrane structure, such as a conformational change as was previously suggested (12,13), may underlie the observed phenomena.
Inhibition of α-methylglucoside uptake in *B. subtilis* cells required the application of distinctly higher concentrations of staphylococcin 1580. This seems to contradict the previously reported inhibition of *o*-nitrophenyl-β-galactoside (ONPG) hydrolysis in *S. aureus*, which is also mediated by the phospho*enol*pyruvate phosphotransferase system (17), by moderate staphylococcin concentrations. However, it may reflect a different regulation of uptake systems in both organisms, rather than the induction of gross permeability changes in *S. aureus*. Moreover, the present results are in accordance with the observations made with colicins E1 and K (14) and Ia (9).

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CHAPTER 9

BIOCHEMICAL AND ULTRASTRUCTURAL CHANGES IN *STAPHYLOCOCCUS AUREUS* TREATED WITH STAPHYLOCOCCIN 1580

INTRODUCTION

The primary effect induced by staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis* 1580, was previously shown to be located on the cell membrane, and is manifested in a collapse of the membrane electrical potential, which occurred after only 20 seconds (26). Concomitantly, a variety of processes, generally connected to the energy metabolism, such as macromolecular synthesis, active transport and maintenance of ion-gradients, appear strongly distorted by the action of the bacteriocin (16). The ultimate death of the cells was suggested to depend on the ability of cells to restore the primary lesions after removal or inactivation of the bacteriocin, and recovery of the primary injury was shown to occur under favorable conditions (25).
The present study was undertaken to reveal biochemical and ultrastructural changes that might be connected to the irreversible lesions induced by staphylococcin 1580.

MATERIALS AND METHODS

Bacterical strain and growth conditions
Staphylococcus aureus Oxford 209P was used throughout this study and was grown aerobically at 37°C in tryptone soya broth (Oxoid) or in semi-synthetic medium A (25) with 1% sodium pyruvate as carbon source, where indicated. Cells from the midlogarithmic phase of growth were washed, and finally resuspended in 50 mM potassium phosphate (pH 7.0) at about 6×10^8 cells per ml.

Production and assay of staphylococcin 1580
The bacteriocin was prepared and assayed as described previously (25). The activity was expressed as arbitrary units per ml.

Viable count

Viable counts after staphylococcin 1580-treatment were determined by rapidly diluting samples 1000-fold into ice-cold tryptone soya broth, and subsequently plating suitably diluted amounts on both tryptone soya agar (TSA) and TSA supplied with 1 M NaCl (TSAS). The latter medium was used to measure the loss of salt tolerance (25). The survival ratio (R_t) was defined as the ratio between the viable counts of treated and untreated suspensions.

Incorporation and release of radioactive substances

DNA, RNA and protein syntheses were studied by measuring the incorporation of [^{3}H]thymidine, [^{3}H]uracil and [^{14}C]glutamic acid, respectively, as described previously (16). Incorporation into trichloroacetic acid (TCA)-precipitable material was measured as described (16). The total uptake of label was determined by filtering adequate cell samples on membrane filters (0.45 µm pore size, Millipore Corp.) and washing with five volumes of the incubation buffer, after which the filters were processed as described (16). The total radioactivity in the incubation medium was measured by counting samples of the filtration fluid in Bray's mixture (4). TCA-precipitable label in the medium was detected by collecting the filtration fluid to which TCA (5% final concentration) was added. The TCA-soluble radioactivity was calculated by difference after conversion of the results to dpm.

The incorporation of lysine into peptidoglycan was measured according to Chmara and Borowski (5).

Fatty acid composition

Total fatty acids were extracted and methylated as described by Dunlap and Perry (9) and were run on a Pye series 104 gas chromatograph, on a 5 ft glass column with 10% diethyleneglycol succinate on 100 to 120 mesh Diatomite C at 170°C, as described previously (24).

Polyacrylamide gel electrophoresis of membrane proteins

Membranes were prepared according to the method described by

Bisschop and Konings (3) except that the pH of all buffers was 7.0 and lysostaphin (final concentration 50 µg per ml) replaced lysozyme. The incubation time with lysostaphin was reduced to 15 min and centrifugation was carried out at 4°C. The membranes were finally suspended in 40 mM Tris·HCl (pH 8.0) at a concentration of 1 mg protein per ml, and stored at -20°C. Protein was measured by the Lowry-method.
Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 10% slab gels was carried out as described by Ames (1) in a Bio-rad model 220 apparatus (Bio-rad Lab. Richmond, Cal.). The membranes were dissolved by boiling for 2 min in the presence of 3% SDS and 2% 2-mercaptoethanol.

Preparation of protoplasts
Protoplast from *S. aureus* cells were prepared by a treatment with lysostaphin (50 µg per ml) for 30 min at 37°C in 50 mM potassium phosphate (pH 7.0) and 24% NaCl (15).

Electron microscopic studies
To staphylococcin 1580-treated or control cell suspensions ($6x10^8$ cell/ml) glutaraldehyde to a final concentration of 0.1% was added, the cells were centrifuged for 2 min at 18.000 x *g* at room temperature and resuspended in sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde. After 30 min the cells were postfixed in 1% OsO_4 in standard Palade buffer for 2 h at room temperature or overnight in the cold. Fixed cells were mixed with 1% agar, cut into 1 cm^3 cubes and dehydrated with aceton series and embedded in Epon 812. Thin sections were cut on a Porter-Blum model MT2-B microtome, and stained with 1% uranyl acetate solution and lead citrate according to the Millonig method.
For freeze-etch preparation cell suspensions were centrifuged for 2 min at 18.000 x *g* at room temperature and rapidly frozen in liquid Freon. The classical method described by Moor *et al* (20) was followed, using a Balzers freeze-etching device (Balzer High Vacuum Corp.) A Philips EM300 electron microscope was used.

Chemicals

L-[^{14}C]glutamic acid (270 mCi/mmol), [^{3}H]thymidine (5 Ci/mmol), [^{3}H]uracil (1 Ci/mmol) and L-[^{14}C]lysine (330 mCi/mmol) were obtained from Radiochemical Centre, Amersham. Lysostaphin was from Schwarz-Mann and acrylamide, methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine from Bio-rad. All other chemicals were of reagent grade.

RESULTS

Effect on macromolecules

It was previously shown that treatment of *S. aureus* with staphylococcin 1580 blocks immediately and independently the syntheses of protein, RNA and DNA, and of glycogen-like material (16). Fig. 1 shows that the incorporation of lysine into cell wall peptidoglycan is also immediately halted by the bacteriocin.

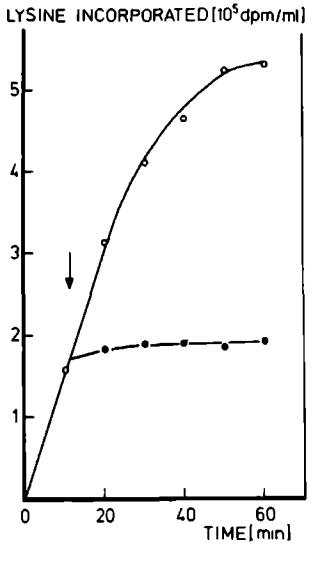

Fig. 1. Effect of staphylococcin 1580 on the incorporation of lysine into peptidoglycan. Mid-exponential phase cells of *S. aureus*, grown on Medium A-pyruvate were washed and resuspended in cell wall synthesis medium I (5) to about 10^9 cells per ml. After 10 min incubation at 37°C in the presence of 100 µg/ml of chloramphenicol, [^{14}C]lysine (2.5 µCi, 330 mCi/mmol) was added. 11 min later, the incubation mixture was split into two portions, one of which received staphylococcin 1580 dissolved in incubation medium (final concentration 200 arbitrary units per ml), the other received an equal amount of incubation medium. At intervals samples were removed, mixed with an equal amount of 10% perchloric acid and kept at 0°C for 30 min after which the radioactivity in the precipitate was determined. Symbols: 0, control; ●, treated with staphylococcin 1580.

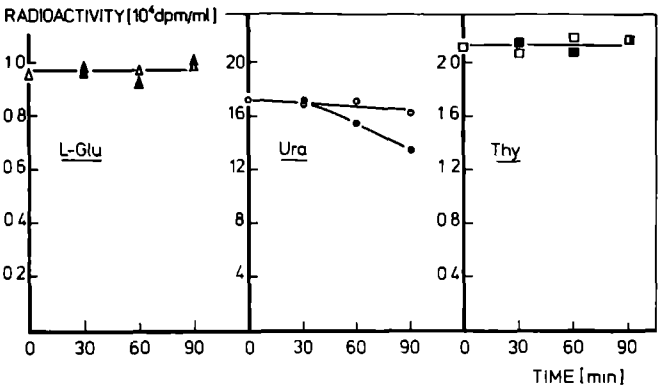

Fig. 2. Effect of staphylococcin 1580 on the integrity of protein, RNA and DNA. *S. aureus* cells, exponentially growing on medium A-pyruvate in the presence of labelled precursors for 2 h were centrifuged, washed and resuspended in the same medium without the labels at a concentration of 8×10^8 cells/ml and incubated at 37°C. The experiments were started by the addition of staphylococcin 1580 (final concentration 200 arbitrary units/ml) dissolved in 50 mM potassium phosphate (pH 7.0); controls received only buffer. At intervals samples were removed and the trichloroacetic acid-insoluble radioactivity was measured. Symbols: Δ, 0, □, controls; ▲, ●, ■, treated with staphylococcin 1580; Δ, ▲, cells prelabelled with L-[^{14}C]glutamic acid (0.5 µCi/ml); 0, ●, prelabelled with [^{3}H]uracil (0.5 µCi/ml); □, ■, prelabelled with [^{3}H]thymidine (0.5 µCi/ml).

The effect of staphylococcin 1580 on the stability of macromolecules was investigated by following the fate of incorporated labelled precursors in cells treated with staphylococcin 1580 and in cells not treated with the bacteriocin.

The results presented in Fig. 2 confirm the conclusions previously drawn in a study on macromolecular synthesis (16). During an incubation period of 90 min no degradation of DNA and protein was observed. However, radioactivity is lost from the TCA-insoluble pool in cells labelled with [^{3}H]uracil, indicating a degradation of RNA.

Table 1 shows an analysis of the label released from the cells in the presence or absence of staphylococcin 1580. A substantial loss of [^{14}C]glutamic acid and [^{3}H]thymidine preaccumula-

TABLE 1. EFFECT OF STAPHYLOCOCCIN 1580 (st1580) ON THE RELEASE OF PREACCUMULATED PRECURSORS OF PROTEIN-, RNA- AND DNA- SYNTHESES

Precursor	Total radioactivity accumulated (dpm/ml)	%	Radioactivity retained in the cell after 30 min (%) -st1580	Radioactivity retained in the cell after 30 min (%) +st1580	Radioactivity released after 30 min (%) TCA soluble -st1580	Radioactivity released after 30 min (%) TCA soluble +st1580	Radioactivity released after 30 min (%) TCA insoluble -st1580	Radioactivity released after 30 min (%) TCA insoluble +st1580
L-glutamic acid	1.5×10^4	100	75	61	24	38	0.5	1
thymidine	3.2×10^4	100	74	73	19	25	7	2
uracil	1.8×10^5	100	88	93	8	6	4	1

S. aureus cells were grown and treated as described in Fig. 2. The intracellular radioactivity and the activity in trichloroacetic acid (TCA)-soluble and TCA-insoluble fractions of the medium was determined as described in Materials and Methods.

ted by the cells (TCA-soluble + TCA-insoluble), was observed after 30 min of incubation, both in the presence and in the absence of the bacteriocin. Staphylococcin 1580 increased the amount of L-glutamic acid released, but only from the TCA-soluble pool, since no degradation of protein occurs (Fig. 2) and no TCA-insoluble material is lost. Staphylococcin 1580 had no significant effect on the release of TCA-soluble substances labelled by either [^{3}H]thymidine or [^{3}H]uracil, but apparently slightly decreased the release of TCA-insoluble material. These results confirm the general integrity of the cell permeability barrier.

Effect on the cell membrane

Staphylococcin 1580 had no marked effect on the protein composition of membranes of cells, and neither on that of isolated membrane preparations (Fig 3). Moreover, the yield of membrane proteins was not affected by the bacteriocin-treatment and amounted to about 3.4% of the dry weight of the cells in all samples tested.

In a previous report (17) it was shown that a rapid shift in the relative amounts of diphosphatidylglycerol and phosphatidylglycerol occurred upon treatment of cells with staphylococcin 1580, but the total phospholipid content of the cells was only slightly affected. We studied the effect of the bacteriocin on the total fatty acid composition of *S. aureus* cells (Table 2). No significant changes could be observed except for a slight increase in the relative amount of the normal C20 acid at the expence of the normal C18 acid.

The effect of staphylococcin 1580 on the stability of protoplasts, prepared with the cell wall-degrading enzyme lysostaphin, is shown in Fig. 4. Although the effect of the bacteriocin in whole cells is nonlytic (15), a distinct drop in the turbidity ($A_{600\,nm}$) of a protoplast suspension prepared from cells taken from the mid-exponential phase of growth, was observed. The extent of the drop suggests that only a fraction of the protoplasts lyses or may reflect morphological changes of the protoplasts. The latter explanation may rather be valid as is shown in the following section. Protoplasts prepared from

TABLE 2. EFFECT OF STAPHYLOCOCCIN 1580 ON THE FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACTS OF *S. AUREUS* CELLS

	Fatty acids (%)	
Fatty acid	not treated	treated with staphylococcin 1580
br-C13:0	1.2	0.8
br-C14:0	2.4	1.5
n-C14:0	4.2	3.2
br-C15:0	34.0	32.4
br-C16:0	0.4	0.2
n-C16:0	6.4	6.1
br-C17:0	5.2	5.1
br-C18:0	0.1	0.2
n-C18:0	20.4	18.2
n-C18:1	4.2	3.6
br-C19:0	0.7	0.6
n-C19:0	2.5	2.9
n-C20:0	14.4	18.1
n-C20:1	0.5	0.6

S. aureus cells were grown in tryptone soya broth into mid-exponential phase, washed and resuspended ($6x10^8$ cells/ml) in 50 mM potassium phosphate (pH 7.0), 50 mM sodium chloride and 10 mM magnesium sulfate and incubated for 30 min at 37°C in the presence or absence of staphylococcin 1580 (150 arbitrary units per ml). Subsequently, the cells were rapidly centrifuged (5 min 18.000 x *g*) and the pellet extracted as described (24). The results are given as the percentage of each fatty acid fraction of the total sum of fractions. Minor fractions (less than 0.1% of total fatty acids) are not shown.
n = straight chain fatty acids
br= methyl branched, iso- and anteiso fatty acids.

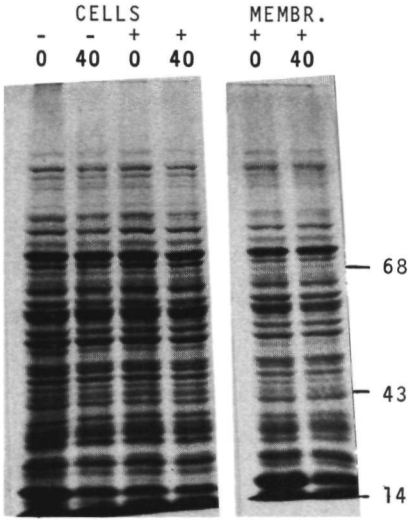

Fig. 3. Electrophoresis of membrane proteins prepared from cells or membranes treated with or without staphylococcin 1580. Cells were grown on medium A with 1% glucose into mid-exponential phase, washed and resuspended in 50 mM potassium phosphate (pH 7.0), 50 mM sodium chloride and 10 mM magnesium sulfate to about 6×10^9 cells per ml. The suspension was divided into two parts, one of which received staphylococcin 1580 to a final concentration of 250 arbitrary units per ml, the other was not treated with the bacteriocin. Incubation was at 37°C. At intervals samples were removed and membranes were prepared as described. The log survival ratios (R_t) after 5 min treatment with staphylococcin 1580 were - 0.70 on TSA and - 2.35 on TSAS. Alternatively, membranes prepared from untreated cells were incubated with staphylococcin 1580 under similar conditions. Markers represent the position of bovine serum albumin (68.000d), yeast alcohol dehydrogenase (43.000d) and ribonuclease (13.700d).

stationary cells were not affected, which agrees well with the increased resistance of these cells to staphylococcin 1580 (26).

Effect on the ultrastructure

Fig. 5 shows representative examples of ultrathin sections of *S. aureus* cells not treated (Fig. 5A) or treated with staphylococcin 1580 for 30 min (Fig. 5B-D). Some pronounced morphological alterations are apparent in the bacteriocin-treated cells. The appearance of large mesosomal structures is observed in al-

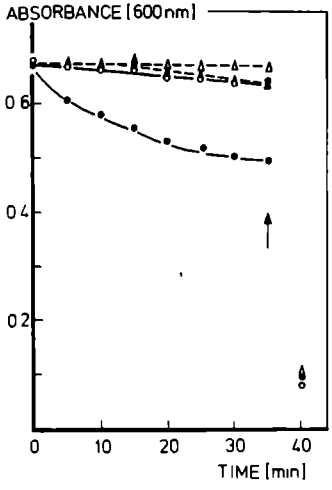

Fig. 4. Effect of staphylococcin 1580 on the turbidity of protoplast suspensions. Protoplasts were prepared from *S. aureus* cells grown on tryptone soya broth by lysostaphin treatment in a medium containing 50 mM potassium phosphate (pH 7.0) and 24% sodium chloride. Incubation was at 37°C. The experiment was started by the addition of staphylococcin 1580 (100 arbitrary units/ml) dissolved in incubation medium, or of an equal volume of this medium. The absorbance at 600 nm was followed. After 35 min of incubation sodium dodecyl sulfate (SDS) to a final concentration of 0.05% was added to lyse the protoplasts. open symbols: not treated with staphylococcin 1580; closed symbols: treated with staphylococcin 1580. ○, ●, protoplasts prepared from mid-exponential phase cells; △, ▲, protoplasts prepared from cells taken from the stationary phase.

most all cells, mostly appearing as honeycomb arrays of tubular form and sometimes as vesicular mesosomes, connected with the cytoplasmic membrane. Almost all treated cells exhibit extensive condensation of cytoplasmic material in the nucleoid area, appearing as densely stained, stacked fibrils, in connection with a clearing of the area. However, the structure of the cytoplasm outside the nucleoid seems not to be changed.

Freeze-fracturing of cells was used to study the effect of staphylococcin 1580 on the ultrastructure of the membrane. The outer fracture-face (convex face) of untreated cells shows a relatively regular distribution of particles (Fig. 6A). After 5 min treatment with staphylococcin 1580 no significant differences were observed (Fig. 6B), although at this time the cells are severely injured by the action of the bacteriocin (25). However, prolonged incubation with staphylococcin 1580 results in a segregation of the particles into particle-dense and undulated,

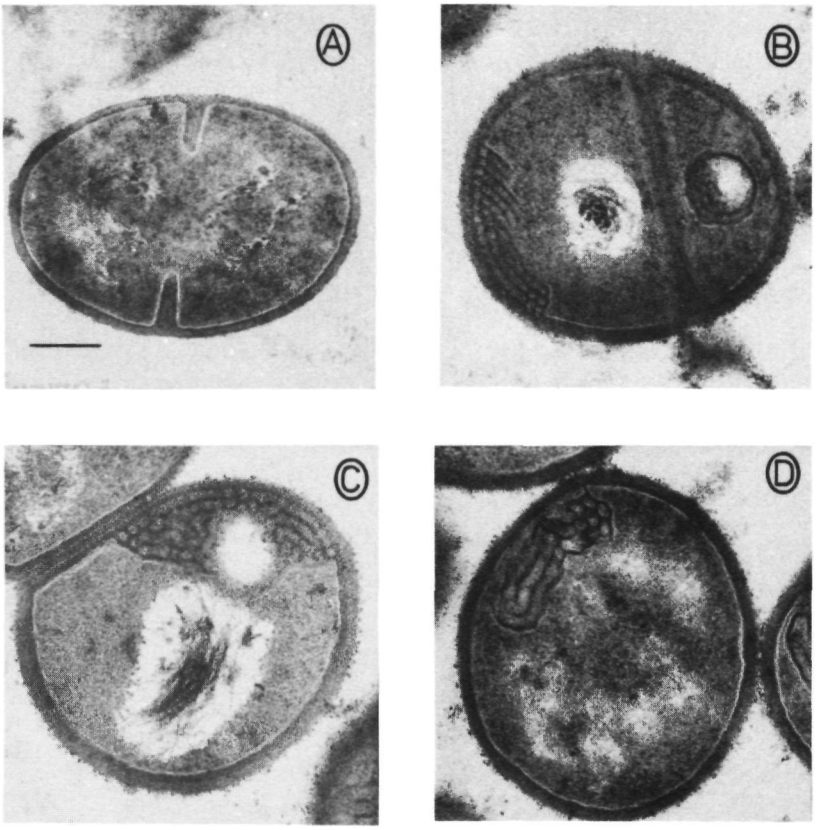

Fig. 5. Thin sections of *S.aureus* cells. Cells from the mid-exponential phase were washed and resuspended ($6x10^8$ cells/ml) in 50 mM potassium phosphate buffer (pH 7.0) and treated with staphylococcin 1580 (100 arbitrary units per ml) (B-D) or only buffer (A), for 30 min at 37°C. Staphylococcin 1580-treated cells show extensive mesosome-like structures and clearing of the nucleoid area.
Bar represents 0.2 µm.

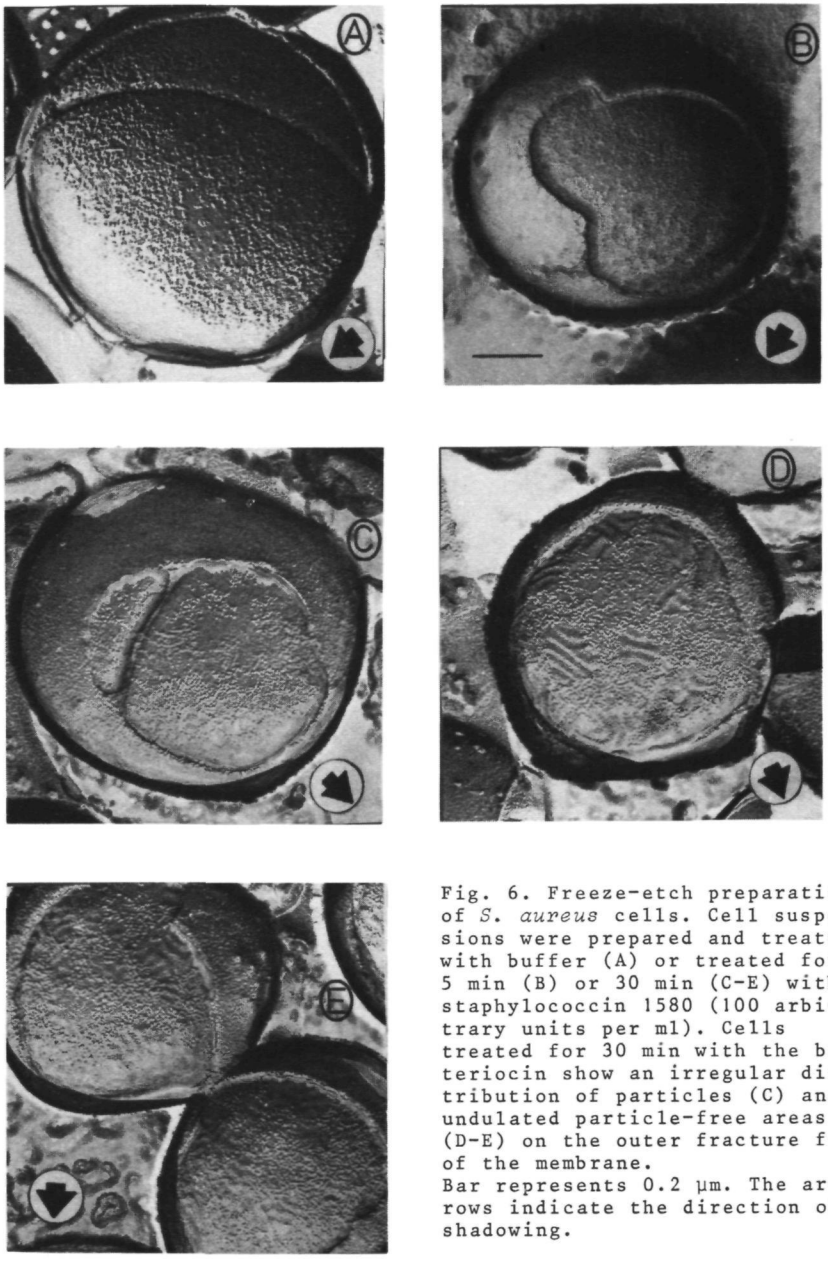

Fig. 6. Freeze-etch preparations of *S. aureus* cells. Cell suspensions were prepared and treated with buffer (A) or treated for 5 min (B) or 30 min (C-E) with staphylococcin 1580 (100 arbitrary units per ml). Cells treated for 30 min with the bacteriocin show an irregular distribution of particles (C) and undulated particle-free areas (D-E) on the outer fracture face of the membrane.
Bar represents 0.2 µm. The arrows indicate the direction of shadowing.

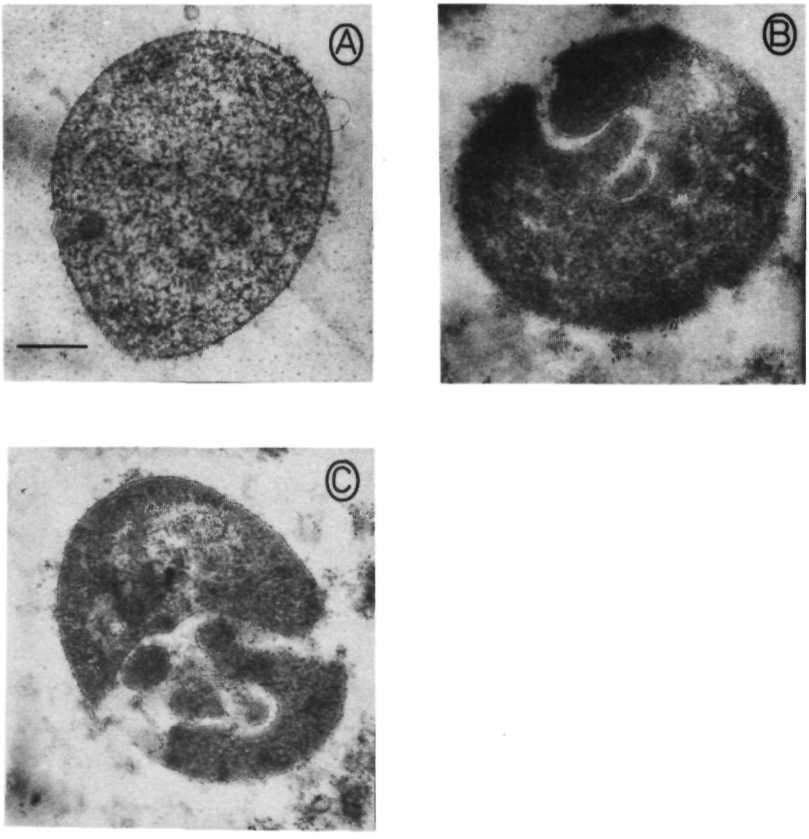

Fig. 7. Thin sections of *S. aureus* protoplasts. Protoplasts were prepared in 50 mM potassium phosphate buffer (pH 7.0) and 24% sodium chloride as described in Materials and Methods, and treated with buffer (A) or staphylococcin 1580 (100 arbitrary units per ml)(B-C) for 30 min at 37°C. Staphylococcin 1580-treated protoplasts show extensive invaginations of the membrane.
Bar represents 0.2 μm.

particle-free areas, in almost all cells (Fig. 6C-E). The latter areas may represent the mesosome-like honeycomb arrays at the outer surface of the plasma membrane since they show a similarity in size and arrangement, and they resemble some of the structures observed in freeze-fractured *Bacillus subtilis* cells, which structures were attributed to mesosomes (21).
The alterations of the membrane ultrastructure resemble in some way the changes observed after cooling membranes below the transition temperature of the membrane lipids, which results in the squeeze out of protein particles due to the close packing of lipids in the crystalline state. The effects induced by staphylococcin 1580 thus may represent a conformational change in the membrane. However, such temperature induced ultrastructural changes have not been found in *S. aureus* (14) probably as a result of the looser packing of the lipids in this organism due to its high content of branched chain fatty acids (14). The amount of branched chain fatty acids was not significantly affected by staphylococcin 1580. Moreover, the above supposed absence of particles in the mesosome-like structures is in accordance with the lipid content of mesosomal membranes, which are reported to contain 2 - 6 times higher amounts of all lipid classes in comparison with plasma membranes in *S. aureus* (2).
Thin sections of protoplasts, prepared from *S. aureus* cells by treatment with lysostaphin, show that morphological alterations evolve from invaginations of the membrane upon treatment with staphylococcin 1580 (Fig. 7B-C). Finally, extensive, empty, membrane-bounded spaces are created. However, the results obtained with protoplasts are probably strongly influenced by the extremely high NaCl-concentration, prerequisite to stabilize the protoplasts (15).

DISCUSSION

The present study focusses on the biochemical and ultrastructural changes which become detectable upon staphylococcin 1580-treatment and concur with the irreversible lesions of the cells. Various results showed a striking similarity to those obtained

with other bacteriocins, and may either indicate similar effects of these bacteriocins, or a similar response of the cells to various kinds of stress applied.

Some degradation of RNA occurs after prolonged exposure to the bacteriocin. This degrading action is shared by a variety of bacteriocins from gram-positive bacteria, such as staphylococcin C55 (7) and enterocin A-FF22 (21). It is not clear yet, if this action is an inherent property of these bacteriocins, or if it reflects activation of a ribonuclease in the cells, possibly through a change in the ionic composition.

Thin sections of staphylococcin 1580-treated cells showed extensive condensation of cytoplasmic material in the nucleoid area but no degradation of DNA was observed. Similar results have been reported for staphylococcin C55 (6), enterococcin A-FF22 (22) and boticin E-$S5_1$ (10). Remarkably, in this respect, are the results obtained by Dajani *et al* (8) with viridin B, a bacteriocin of α-hemolytic streptococci, which is bactericidal to *Neisseria sicca* and concomittantly induces condensations in the nucleoid area, but only bacteriostatic against a coagulase-negative *Staphylococcus* in which only ultrastructural alterations in the mesosomes were observed.

No major lesions in the cytoplasmic membrane could be observed, which suggests that the primary changes in the permeability of cells might rather result from subtle and local changes of the membrane conformation than from gross structural alterations. Moreover, the general integrity of the membrane remains intact during staphylococcin 1580-treatment, since cells retain the ability to concentrate α-methylglucoside (Chapter 8). In contrast to the effect exerted by colicins E1 and K (20) no specific proteins were lost from the membranes of cells upon treatment with staphylococcin 1580. However, protein synthesis is needed for the recovery of sublethally injured cells (25), which suggests that damaged components of the cell must be replaced. The effect of staphylococcin 1580 on the fatty acid composition of the membrane is insignificant and the increase in diphosphatidylglycerol at the expence of phosphatidylglycerol (17) is not likely the cause of the increased salt sensitivity of the treated cells, but may be a result of an attempt to

repair salt tolerance, since increased salt tolerance in *S. aureus* is generally attended with increased diphosphatidylglycerol contents (23). The development of extensive mesosome-like structures upon treatment with staphylococcin 1580 may also be a reflection of an attempt to repair primary damage of the cells, since it has been suggested that mesosomes in *S. aureus* are involved in lipid synthesis (2). Molenkamp and Veerkamp (19), in a study on the effect of various growth inhibitors on the cell envelope of *Bifidobacterium bifidum*, found that staphylococcin 1580 increased the incorporation and turnover of oleic acid and the release of lipids from the cells of this bacterium.

The increase in mesosomal structures seems to be a commom response to the action of bacteriocins interfering with the energy metabolism of the cell, and was observed upon treatment with staphylococcin C55 (6), enterocin A-FF22 (22), viridin B (8) and boticin $S5_1$ (10), but also as a result of various kinds of stress applied to cells (see 13) and of chemical fixation (11, 12).

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SUMMARY

Bacteriocins are proteinaceous bactericidal substances produced by bacteria. This thesis presents the results of a study of the properties, mode of action and ecological aspects of bacteriocins produced by staphylococci and oral streptococci.

The introduction deals with the general concepts developed in the study of bacteriocins, and reviews the recent literature. During the last decades the study of bacteriocins has largely moved to the molecular level, and added many intriguing features to our understanding of bacterial genetics, physiology and ecology.

Chapters 2 and 3 deal with a study of ecological aspects of bacteriocin production. Bacteriocin-like substances are produced by many streptococci present in human dental plaque, in particular by *Streptococcus mutans*, and act on other streptococcal species. A model system, which allows the study of the interaction of plaque strains *in vitro*, showed that bacteriocin-producing strains are capable to interfere with, and to displace other strains from plaque as a direct result of the antagonistic entity they produce. Comparison of the mutually antagonistic properties of strains isolated from small samples of human dental plaque suggests that such a mechanism might be involved in the *in vivo* regulation of the bacterial flora on a micro-ecological scale.

Chapters 4 to 9 describe experiments carried out with staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis* 1580. Its production is maximal in the late-exponential and early-stationary phase of growth, and strongly dependent on the pH of the medium, which in turn depends on the carbon source applied. A semi-synthetic medium with pyruvate as carbon source formed the basis of a novel production and purification procedure.

The action of staphylococcin 1580 is strongly dependent on the energized state of the target cells, since energy-depleted cells are resistant to the bacteriocin but become rapidly injured when a suitable energy source is added. The reinitiation of the action of staphylococcin 1580 is strongly depressed under con-

ditions which prevent the restoration of high ATP- and NADH-levels in the cell; the energized state of the membrane appears less important in this process. It is concluded that the sensitivity to staphylococcin 1580 is under control of an energy regulating mechanism. In accordance with these observations, the sensitivity of non-starved cells is dependent on their metabolic state and may rapidly shift during growth. Also the temperature of growth and incubation of the cells, and growth conditions which lead to an increased proportion of unsaturated fatty acids in the membrane, affect the sensitivity to the bacteriocin.

The primary effect of staphylococcin 1580 is exerted on the cell membrane: within 20 sec after the addition of the bacteriocin the electrical potential ($\Delta\psi$) is partially abolished, the permeability for monovalent cations changes, the active transport coupled to the membrane potential is inhibited, preaccumulated solutes leak from the cells, the intracellular ATP level decreases and macromolecular syntheses are inhibited. The pH-gradient (ΔpH) over the membrane and the functioning of the respiratory chain are only slightly altered. The uptake of α-methylglucoside, which is coupled to the phospho*enol*pyruvate phosphotransferase system, is even enhanced in the presence of staphylococcin 1580. Thus the general integrity of the permeability barrier is not affected.

Membrane vesicles prepared from susceptible bacteria are also sensitive to the bacteriocin. A comparison of the effects exerted on permeability, active transport and energized state of the membrane in cells and membrane vesicles shows that at least three classes of effects may be distinguished on basis of their sensitivity to the bacteriocin.

Killing of *S. aureus* cells treated with the bacteriocin is dependent on the salt concentration and the alkalinity of the medium. An inverse relationship between the diameter of the cation applied and its promoting effect on killing is observed. These observations suggest that killing results from the inability of cells to restore a high-energy state of the membrane. Under optimal conditions staphylococcin 1580-induced injury may be repaired, which requires a suitable energy source and *de*

novo protein synthesis.

Relatively short incubation of cells with staphylococin 1580 does not induce biochemically and ultrastructurally detectable lesions, although the permeability of the cells is strongly impaired. The primary effect of the bacteriocin thus consists of conformational, rather than structural changes. Prolonged incubation induces definite ultrastructural alterations in the cell membrane and nucleoid area, and degradation of ribonucleic acid.

S A M E N V A T T I N G

Bacteriocines zijn eiwitachtige stoffen die door bacterien geproduceerd worden en dodend werken op andere bacterien. Dit proefschrift bevat de resultaten van een onderzoek naar de eigenschappen, werkingswijze en ecologiese aspekten van bacteriocines die gemaakt worden door staphylococcen en streptococcen, welke laatste geisoleerd zijn uit de mondholte.
De inleiding bevat een overzicht van de belangrijkste punten die in het onderzoek naar bacteriocines naar voren zijn gekomen en een samenvatting van de recente literatuur. Gedurende het laatste tiental jaren is het onderzoek van bacteriocines voor een groot deel verschoven naar het moleculaire vlak, en heeft het belangrijke bijdragen geleverd aan het begrijpen van de bacteriele genetica, fysiologie en ecologie.
In Hoofdstuk 2 en 3 zijn ecologiese aspekten van bacteriocineproduktie behandeld. Veel streptococcen die in de menselijke tandplak voorkomen, in het bijzonder *Streptococcus mutans*, produceren bacteriocine-achtige stoffen die werkzaam zijn tegen andere streptococcen. Met behulp van een modelsysteem dat het mogelijk maakt *in vitro* de interakties van plak mikroorganismen te bestuderen werd aangetoond dat bacteriocine-producerende stammen in staat zijn met behulp van het bacteriocine andere stammen uit de plak te verdringen. Aanwijzingen dat een dergelijk mechanisme *in vivo* een rol zou kunnen spelen bij de regulatie van de bacteriele samenstelling op een micro-ecologiese schaal, werden verkregen door vergelijking van de wederzijdse remmende invloeden van stammen in kleine plak monsters.
In Hoofdstuk 4 tot en met 9 zijn experimenten beschreven die gedaan werden met staphylococcine 1580, een bacteriocine dat geproduceerd wordt door *Staphylococcus epidermidis* 1580. De aanmaak van het bacteriocine is maximaal in de eindfase van de groei, en sterk afhankelijk van de zuurgraad (pH) van het medium, dat op zijn beurt weer afhangt van de koolstofbron die gebruikt is. Een semi-syntheties medium met pyruvaat als koolstofbron werd gekozen als uitgangspunt voor een nieuwe produktie- en zuiveringsmethode.
De werking van het staphylococcine1580 is sterk afhankelijk van

de energetiese toestand van de cellen waarop het aangrijpt. Cellen waarvan de energievoorraden opgebruikt zijn, zijn ongevoelig voor het bacteriocine, maar worden snel aangetast wanneer een geschikte energiebron wordt toegevoegd. Dit herstel van de gevoeligheid is sterk onderdrukt onder omstandigheden waarbij de vorming van hoge ATP en NADH nivo's voorkomen wordt. Een energierijke toestand van de celmembraan lijkt in dit proces van minder belang. Dit leidt tot de konklusie dat de gevoeligheid voor staphylococcine 1580 gekontroleerd wordt door een mechanisme dat de energiehuishouding in de cel regelt. Hiermee in overeenstemming is de waarneming dat de gevoeligheid van niet-gehongerde cellen afhangt van hun metabole toestand, die sterk kan veranderen gedurende de groei. Bovendien hebben de groei- en inkubatietemperatuur en omstandigheden die leiden tot een verhoogd gehalte aan onverzadigde vetzuren invloed op de gevoeligheid voor het bacteriocine.

Staphylococcine 1580 oefent zijn invloed primair uit op de celmembraan: 20 seconden na toevoegen van het bacteriocine wordt de elektriese potentiaal ($\Delta\psi$) gedeeltelijk teniet gedaan, de permeabiliteit voor eenwaardige kationen verandert, aktief transport gekoppeld aan de membraan potentiaal wordt geremd, reeds opgehoopte stoffen lekken weer naar buiten, het ATP nivo in de cel daalt en de syntheses van macromoleculen wordt geremd. De pH-gradient (ΔpH) over de celmembraan en de ademhaling van de cel worden echter nauwelijks beinvloed. De opname van α-methylglucoside, die gekoppeld is aan het fosfo*enol*pyruvaat fosfotransferase systeem, wordt zelfs gestimuleerd door staphylococcine 1580. De permeabiliteitsbarriere van de cel blijft dus intakt.

Ook membraan blaasjes, die gemaakt zijn van staphylococcine 1580-gevoelige cellen, zijn gevoelig voor het bacteriocine. De effekten op permeabiliteit, aktief transport en energie toestand kunnen in tenminste drie klassen ingedeeld worden voor wat betreft hun gevoeligheid voor staphylococcine 1580.

De afdoding van cellen door staphylococcine 1580 is afhankelijk van de zoutkonsentratie en de zuurgraad van het medium waarin deze cellen na behandeling gebracht worden. De mate van afdoding blijkt omgekeerd evenredig te zijn met de straal van het

gebruikte kation. Deze resultaten suggereren dat de cellen afgedood worden doordat ze niet meer in staat zijn de energierijke toestand van hun membraan te herstellen. De door staphylococcine 1580 aangebrachte beschadigingen kunnen onder optimale omstandigheden hersteld worden, waarvoor een geschikte energiebron en de aanmaak van eiwitten noodzakelijk zijn.
Wanneer cellen maar betrekkelijk kort met staphylococcine 1580 behandeld zijn, kunnen geen biochemiese of ultrastrukturele veranderingen worden aangetoond, hoewel hun permeabiliteit dan al sterk beinvloed is. Het is dus waarschijnlijker dat het primaire effekt van staphylococcine 1580 uit conformatie veranderingen dan uit strukturele veranderingen bestaat. Behandeling gedurende langere tijd leidt wel tot duidelijke, met behulp van een elektronenmikroskoop waarneembare veranderingen in zowel de celmembraan als in het nucleoid, en tot afbraak van ribonucleine zuur.

C U R R I C U L U M V I T A E

De schrijver van dit proefschrift werd geboren te Enschede op 29 april 1947. Hij behaalde het einddiploma H.B.S.-B aan het St. Jacobuscollege aldaar in 1965 en begon in datzelfde jaar zijn biologie studie aan de Katholieke Universiteit te Nijmegen. Het doctoraalexamen met hoofdvak Exobiologie (Dr. W. Heinen) en bijvakken Biochemie (Prof. Dr. H. Bloemendal) en Chemiese Dierfysiologie (Dr. A. M. Th. Beenakkers), werd behaald in maart 1972. Gedurende twee jaar was hij part-time verbonden als leraar biologie aan middelbare scholen te Nijmegen en Boxtel. Sinds maart 1973 is hij werkzaam op de afdeling microbiologie van deze Universiteit.

S T E L L I N G E N

I

De door *Brummett* en *Ordal* gehanteerde bewijsvoering voor hun stelling dat ontkoppelaars op specifieke wijze reageren met transportcarriers is onjuist, omdat voorbijgegaan wordt aan de wijze waarop koppeling van energie aan het transportsysteem plaats vindt.

T.B. Brummett en G.W. Ordal (1977) Arch. Biochem. Biophys. 178: 368-372

II

De effekten van colicine K op de fluorescentie van 3,3'-dihexyloxacarbocyanine in *Escherichia coli* cellen, kunnen even goed verklaard worden door de invloed van dit bacteriocine op het gehalte en de verdeling van magnesium ionen, dan door effekten op de membraanpotentiaal.

G.J. Brewer (1976) Biochemistry 15: 1387-1392

III

Om meerdere redenen dient het gebruik van antibiotica beperkt te worden, en waar mogelijk vervangen door de ecologies meer zinvolle toepassing van antagonistiese microorganismen.

R. Aly, H.I. Maibach, H.R. Shinefield, A. Mandel en W.G. Strauss (1974) J.Infect. Dis. 129: 720-724
C.C. Sanders, W.E. Sanders jr. en D.J. Harrowe (1976) Infect.Immun. 13: 808-812

IV

De inspanningen om kariësprofylaxe te verkrijgen door een gerichte bestrijding van *Streptococcus mutans* zijn tevergeefs, aangezien wél *S.mutans* maar niet kariës bestreden wordt.

V

De experimenten van *von der Helm* bewijzen geenszins dat het Rous sarcoma viraal eiwit p15 betrokken is bij de proteolytiese splitsing van de polypeptide precursor Pr76.

K. von der Helm (1977) Proc.Natl.Acad. Sci. U.S.A. 74:911-915

VI

De voorstellen voor een afzonderlijke studierichting Biochemie miskennen het belang van een wezenlijke biologiese inbreng in de biochemie, en werken mee aan de vergroting van de toch al voelbare kloof tussen biologen en biochemici.

Voorstellen Wet Herstrukturering Wetenschappelijk Onderwijs

VII

Het standaardmodel voor het door de rijksoverheid aan een groot aantal gemeenten opgelegde onderzoek naar de huisvesting van alleenstaanden en tweepersoonshuishoudens biedt nauwelijks mogelijkheden om tot verantwoorde indikaties te komen inzake het gewenste aandeel van de kleinere woningen in de totale woningproduktie.

VIII

Ten onrechte zijn vele leden van de universitaire gemeenschap zich onvoldoende bewust van hun maatschappelijke verantwoordelijkheid.

IX

Het bezigen van de uitdrukking "milieuvriendelijk", terwijl "minder schadelijk" bedoeld wordt, is symptomaties voor een gevoel van onverplichte goedheid ten opzichte van het milieu.

Nijmegen,
2 september 1977

A.H. Weerkamp