The Genera *Staphylococcus* and *Macrococcus*

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**Introduction**

The name *Staphylococcus* (staphyle, bunch of grapes) was introduced by Ogston (1883) for the group micrococci causing inflammation and suppuration. He was the first to differentiate two kinds of pyogenic cocci: one arranged in groups or masses was called “*Staphylococcus*” and another arranged in chains was named “Billroth’s *Streptococcus*.” A formal description of the genus *Staphylococcus* was provided by Rosenbach (1884). He divided the genus into the two species *Staphylococcus aureus* and *S. albus*. Zopf (1885) placed the mass-forming staphylococci and tetrad-forming micrococci in the genus *Micrococcus*. In 1886, the genus *Staphylococcus* was separated from *Micrococcus* by Flügge (1886). He differentiated the two genera mainly on the basis of their action on gelatin and on relation to their hosts. Staphylococci liquefied gelatin and were parasitic or pathogenic or both whereas micrococci were variable in their action on gelatin and were saprophytic. The genera *Staphylococcus, Micrococcus* and *Planococcus*, containing Gram-positive, catalase-positive cocci, were later placed in the family Micrococcaceae. Evans et al. (1955) proposed separating staphylococci from micrococci on the basis of their relation to oxygen. The facultative anaerobic cocci were placed in the genus *Staphylococcus* and the obligate aerobic cocci in the genus *Micrococcus*. By the mid-1960s, a clear distinction could be made between staphylococci and micrococci on the basis of their DNA base composition (Silvestri and Hill, 1965). Members of the genus *Staphylococcus* have a DNA G+C content of 33–40 mol%, whereas members of the genus *Micrococcus* have a high G+C content of around 70 mol%. Further studies have shown that staphylococci can be distinguished from micrococci and other catalase-positive cocci on the basis of their cell wall composition (Schleifer and Kandler, 1972; Endl et al., 1983), cytochrome profile (Fallier et al., 1980) and menaquinone pattern (Collins and Jones, 1981), susceptibility to lysostaphin and erythromycin (Schleifer and Kloos, 1975b), bacitracin (Falk and Guering, 1983), and furazolidone (Baker, 1984). Comparative immunochemical studies of catalases (Schleifer, 1986), DNA-DNA hybridization studies, DNA-rRNA hybridization studies (Schleifer et al., 1979; Kilpper et al., 1980), and comparative oligonucleotide cataloguing of 16S rRNA (Ludwig et al., 1981) clearly demonstrated the epigenetic and genetic difference of staphylococci and micrococci. Members of the genus *Staphylococcus* form a coherent and well-defined group of related species that is widely divergent from those of the genus *Micrococcus*. Until the early 1970s, the genus *Staphylococcus* consisted of three species: the coagulase-positive species *S. aureus* and the coagulase-negative species *S. epidermidis* and *S. saprophyticus*, but a deeper look into the chemotaxonomic and genotypic properties of staphylococci led to the description of many new staphylococcal species. Currently, 36 species and several subspecies are recognized in the genus *Staphylococcus* (Table 1).

The genus *Macrococcus* has been described on the basis of comparative 16S rRNA analysis, DNA-DNA hybridization studies, ribotype patterns, cell wall composition, and phenotypic characteristics (Kloos et al., 1998a). Macrococci can be separated from staphylococci on the basis of a generally higher DNA G+C content (38–45 mol%), absence of cell wall teichoic acids (with the possible exception of *M. caseolyticus*), unique ribotype patterns, and generally larger cells. Members of the genus *Macrococcus* are also oxidase positive whereas most staphylococci (exceptions: *S. lentus, S. sciuri* and *S. vitulus*) are oxidase negative. There are four species in the genus *Macrococcus* (Table 1).

**Isolation Techniques**

Isolation of *S. aureus* from Foods

*Staphylococcus aureus* has been confirmed to be the causative agent of many cases of severe food poisoning; therefore, its presence in foods is of major concern. *Staphylococcus aureus* is very susceptible to heat treatment and most sanitiz-
Table 1. List of species and subspecies in the genera *Macrococcus* and *Staphylococcus*.

| Species name | References |
|--------------|------------|
| *Macrococcus bovis* | Kloo et al., 1998a |
| *M. carnosus* | Kloo et al., 1998a |
| *M. caseolyticus* | Schleifer et al., 1982 |
| *M. equiperican* | Kloo et al., 1998a |
| *Staphylococcus arlettae* | Schleifer et al., 1984 |
| *S. auricularis* | Kloo and Schleifer, 1983a |
| *S. aureus* | De la Fuente et al., 1985 |
| *S. aureus subsp. novobiosepticus* | Rohsh Bannerman and Kloo, 1991 |
| *S. capitis* | Kloo and Schleifer, 1975b |
| *S. capitis subsp. capitis* | Bannerman and Kloo, 1991 |
| *S. capitis subsp. urealyticus* | Kloo and Schleifer, 1975b |
| *S. carrai* | Devriese et al., 1983 |
| *S. carnosus* | Schleifer and Fischer, 1982 |
| *S. carnosus subsp. carnosus* | Probst et al., 1998 |
| *S. carnoso subsp. utilis* | Probst et al., 1998 |
| *S. chleiferogens* | Devriese et al., 1978 |
| *S. cohnii* | Kloo and Wolfshohl, 1983b |
| *S. cohnii subsp. cohnii* | Kloo and Wolfshohl, 1983b |
| *S. condimenti* | Probst et al., 1998 |
| *S. delphini* | Varaldo et al., 1983 |
| *S. epidermidis* | Winslow and Winslow, 1908 |
| *S. equorum* | Schleifer et al., 1984 |
| *S. felis* | Igimi et al., 1989 |
| *S. fleuretii* | Verwoz-Rozand et al., 2000 |
| *S. gallinarum* | Devriese et al., 1983 |
| *S. haemolyticus* | Schleifer and Kloo, 1975c |
| *S. hominis* | Kloo and Schleifer, 1975b |
| *S. hominis subsp. hominis* | Kloo et al., 1998b |
| *S. hominis subsp. novobiosepticus* | Kloo et al., 1998b |
| *S. hyicus* | Devriese et al., 1978 |
| *S. intermedius* | Häjek, 1976a |
| *S. kloosii* | Schleifer et al., 1984 |
| *S. lugdunensis* | Freney et al., 1988 |
| *S. lutrae* | Foster et al., 1997 |
| *S. muscae* | Häjek et al., 1992 |
| *S. pasteur* | Chesneau et al., 1983 |
| *S. piscifermentans* | Tanasupawat et al., 1992 |
| *S. pulverei (= *S. vitalinus*)* | Petriko, 1998 |
| *S. saccharolyticus* | Zakrzewska-Czerwinska et al., 1995 |
| *S. saprophyticus* | Klipper-Balz and Schleifer, 1981 |
| *S. saprophyticus subsp. bovis* | Häjek et al., 1996 |
| *S. saprophyticus subsp. saprophyticus* | Häjek et al., 1996 |
| *S. schleiferi* | Freney et al., 1988 |
| *S. schleiferi subsp. coagulans* | Igimi et al., 1990 |
| *S. schleiferi subsp. schleiferi* | Igimi et al., 1990 |
| *S. sciuri* | Kloo et al., 1976a |
| *S. sciuri subsp. carnaticus* | Kloo et al., 1997 |
| *S. sciuri subsp. lentus* | Kloo et al., 1997 |
| *S. sciuri subsp. rodentium* | Kloo et al., 1997 |
| *S. simularis* | Kloo and Schleifer, 1975b |
| *S. succinus* | Lambert et al., 1998 |
| *S. vitalinus* | Webster et al., 1994 |
| *S. xyllosus* | Kloo and Schleifer, 1975b |

Abbreviation: T, type species.

First description.

These agents, when it or its enterotoxins are found in processed foods, poor sanitation is usually indicated. Detailed procedures for preparing food samples for analysis, isolating and enumerating *S. aureus*, and detecting staphylococcal enterotoxins in foods can be found in the following texts: *Compendium of Methods for the Microbiological Examination of Foods* (Downes and Ito, 2001), *Official Methods of Analysis of AOAC International* (Horowitz, 2000), and *Bacteriological Analytical Manual* (BAM; United States Food and Drug Administration, 1995). In addition to the AOAC (Association of Official Analytical Chemists) approved microslide test method (Horowitz, 2000), it is possible to detect enterotoxins directly in culture and in contaminated foods by the following rapid methods: radioimmunoassay (RIA) (Miller et al., 1978), enzyme-linked immunosorbent assay (ELISA), and reverse passive latex agglutination (RPLA). An ELISA kit, available from Tecra Diagnostics (Roseville, Australia), is distributed by International Bioproducts, Inc. (Redmond, WA, USA), and an RPLA is available from Oxoid (Columbia, MD, USA). Molecular methods are being investigated for their usefulness in detecting staphylococcal enterotoxin. For example, Western immunoblotting has been used to detect staphylococcal enterotoxin A (Rasooly and Rasooly, 1998). In this procedure, the staphylococcal enterotoxin A (native or heat-denatured) is separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a membrane and then the membrane is probed with antibodies. The polymerase chain reaction (PCR) is an additional molecular method being studied for its ability to detect *S. aureus* and staphylococcal enterotoxins from food samples (McLaughlin et al., 2000; Atanassova et al., 2001; Tamarapu et al., 2001). One potential problem for PCR is the possibility of false-negative reactions due to PCR inhibitors that might be present in some foods.

Several conventional procedures for isolating *S. aureus* from foods are described below.

**Nonselective Enrichment Procedures** It is often necessary to use nonselective enrichment procedures for the detection of *S. aureus* in processed foods, especially when it is suspected that the food contains a small number of cells that may have been injured, e.g., as a result of heating, freezing, desiccation, or storage, and whose growth could be inhibited by toxic components of a selective enrichment media. The following nonselective (repair) enrichment procedure is appropriate for this use:

Nonselective Enrichment of *S. aureus* (Downes and Ito, 2001).
Transfer a 50-ml aliquot of a 1:10 dilution of the food sample homogenate to 50 ml of double-strength trypticase soy broth (TSB). Incubate the preparation for 3 h at 35–37°C. Then add 100 ml of a single-strength TSB containing 20% NaCl. Incubate for 24 ± 2 h at 35–37°C. Transfer 0.1-ml aliquots of the culture to each of duplicate Baird-Parker agar plates, and spread the inoculum, so as to obtain isolated colonies. Incubate the inoculated plates for 46 ± 2 h at 35–37°C. Select two or more colonies suspected to be S. aureus from each plate. Staphylococcus aureus colonies are usually 1.5 mm in diameter, jet-black to dark gray, smooth, convex, have entire margins and off-white edges, and may show an opaque zone and/or a clear halo extending beyond the opaque zone. Test selected colonies for coagulase activity. Results should be reported as S. aureus present or absent in 5 g of food, following the results of coagulase testing or clumping factor testing.

Coagulase Test (Horowitz, 2000; Downes and Ito, 2001).

Transfer colonies to tubes containing 0.2 ml of brain heart infusion (BHI) broth. Incubate culture suspensions 18–24 h at 35–37°C. Add 0.5 ml of reconstituted coagulase plasma with ethylenediaminetetraacetic acid (EDTA) and mix thoroughly. (The plasma is reconstituted according to manufacturer’s directions. If not available, desiccated coagulase plasma (rabbit) is reconstituted and disodium dihydrate EDTA is added to final concentration of 0.1% in reconstituted plasma.) Incubate at 35–37°C and examine periodically during a 6-h interval for clot formation. Any degree of clot formation is considered a positive reaction. Small or poorly organized clots may be observed by gently tipping tube so that liquid portion of reaction mixture approaches lip of tube; clots will protrude above liquid surface. Coagulase-positive cultures are considered to be S. aureus. Test positive and negative controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37°C for > 18 but ≤ 48 h.

This procedure is recommended by the AOAC for the identification of S. aureus isolated from foods. With this procedure, false-positive tests may occur with mixed cultures, but this will probably be avoided if only well-isolated colonies typical of S. aureus are chosen. On rare occasions, coagulase-negative mutants of S. aureus may be present in foods and overlooked by the above procedures, and the presence of other coagulase-positive staphylococci, such as S. intermedius and certain strains of S. hyicus, may be misrepresented as S. aureus using the coagulase test alone.

Baird-Parker agar base when supplemented with egg yolk tellurite enrichment is recommended for the detection and enumeration of coagulase-positive staphylococci in foods.

### Baird-Parker Agar

| Basal medium:               |          |
|----------------------------|----------|
| Tryptone                   | 10.0 g   |
| Beef extract               | 5.0 g    |
| Yeast extract              | 1.0 g    |
| Glycine                    | 12.0 g   |
| Lithium chloride · 6H2O    | 5.0 g    |
| Agar                       | 20.0 g   |

This basal medium may be special-ordered from Difco Laboratories, Detroit, MI. Suspend ingredients in 950 ml distilled water. Boil to dissolve completely. Dispense 95.0 ml portions in screw-capped bottles. Autoclave 15 min at 121°C. Adjust final pH to 6.8–7.2 at 25°C.

### Egg Yolk Tellurite Enrichment

Soak eggs in aqueous mercuric chloride 1:1000 for not less than one min. Rinse in sterile water and dry with a sterile cloth. Aseptically crack eggs and separate whites and yolks. Blend yolk and sterile physiological saline solution (3 + 7 v/v) in high-speed sterile blender for 5 s. Mix 50.0 ml of blended egg yolk with 10.0 ml of filter-sterilized 1% potassium tellurite. Mix and store at 2–8°C. Bacto egg-tellurite enrichment is a commercial preparation available from Difco Laboratories.

### Preparation of Plates

Add 5.0 ml of prewarmed (45–50°C) enrichment to 95 ml of melted basal medium, which has been adjusted to 45–50°C. Mix well (avoiding bubbles), and pour 15.0–18.0 ml into sterile 15 × 100 mm Petri dishes. Plates can be stored at 2–8°C in plastic bags for 4 weeks. Immediately prior to use, spread 0.5 ml per plate of 20% solution of (membrane) filter-sterilized sodium pyruvate and dry plates at 50°C for 2 h or 4 h at 35°C with agar surface uppermost.

### Selective Enrichment Procedures

Selective enrichment is recommended for raw food ingredients and unprocessed foods expected to contain < 100 S. aureus cells/g and a large population of competing species. The recommended procedure of the AOAC is widely accepted and uses the most probable number technique.

Most Probable Number (MPN) Technique (Horowitz, 2000).

Inoculate three tubes of trypticase soy broth with 10% NaCl and 1% sodium pyruvate at each test dilution with 1-ml aliquots of decimal dilutions of sample. Highest dilution of sample must give a negative endpoint. Incubate 48 h ± 2 h at 35°C. Using 3-mm loop, transfer 1 loopful from each tube showing growth to dried Baird-Parker medium. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate 48 h at 35–37°C. For each plate showing growth, transfer 1 colony suspected to be S. aureus to BHI broth. With sterile needle, transfer
colonies to tubes containing 0.2 ml of brain heart infusion (BHI) broth and to agar slants containing any suitable maintenance medium, e.g., tryptase soy agar, standard plate-count agar, etc. Incubate BHI culture suspensions and slants 18–24 h at 35°C. The BHI culture suspensions are used as inocula for the coagulase test (described above), and the slant cultures are used for ancillary tests or repeats of the coagulase test, if results are questionable. Report S. aureus as MPN/g, according to tables of MPN values (United States Food and Drug Administration, 1995).

Direct Surface Plating Procedures These procedures are sometimes preferred over the MPN technique for the detection of S. aureus in raw or unprocessed foods as they are more rapid and are regarded by some investigators to be more accurate than MPN.

Surface Plating Procedure for the Enumeration of S. aureus (Horowitz, 2000) For each dilution to be plated, aseptically transfer 1 ml of sample suspension to triplicate plates of Baird-Parker agar and distribute the 1 ml of inoculum equally over the triplicate plates (e.g., 0.4, 0.3, and 0.3 ml). Aseptically, spread the inoculum over the surface of the agar. Avoid the extreme edges of the plate. Maintain the plates in an upright position until the inoculum is absorbed by the medium (about 10 min on properly dried plates). If the inoculum is not readily absorbed, plates may be placed in an incubator in an upright position for about 1 h before inverting. Invert plates and incubate 45–48 h at 35–37°C. Select plates containing 20–200 colonies unless plates at only lower dilutions (> 200 colonies) have colonies with the typical appearance of S. aureus. If several types of colonies appear to be S. aureus, count the number of colonies of each type and record counts separately. When plates at the lowest dilution plated contain < 20 colonies, they may be used. If plates containing > 200 colonies have colonies with the typical appearance of S. aureus and typical colonies do not appear on plates at higher dilutions, use these plates for enumeration of S. aureus, but do not count non-typical colonies. Select one or more colonies of each type counted and test for coagulase production. Coagulase-positive cultures may be considered to be S. aureus. Add the number of colonies on triplicate plates represented by colonies giving a positive coagulase test, and multiply the total by the same dilution factor. Report this number as S. aureus per gram of product tested. The sensitivity of this procedure may be increased by using larger volumes (> 1 ml) distributed over > 3 replicate plates. Plating of two or more decimal dilutions may be required to obtain plates with the desired number of colonies per plate.

Direct enumeration of coagulase-positive S. aureus can be made on Baird-Parker agar containing rabbit plasma-fibrinogen tellurite (Boothby et al., 1979) or Baird-Parker agar without egg yolk to which a tempered pork plasma-fibrinogen overlay agar has been added (Hauschild et al., 1979). In place of Baird-Parker agar, some laboratories have reported the successful use of tellurite polymyxin egg yolk agar (Crisley et al., 1964), Kaliumrhodanid (i.e., potassium thiocyanate)-Actidione-Natriumzid (i.e., sodium azide)-Egg yolk-Pyruvate (KRA-NEP) agar (Sinell and Baumgart, 1966), and Schleifer-Krämer (SK) agar (Schleifer and Krämer, 1980) for the selective isolation and enumeration of staphylococci from foods.

SK Agar for Selective Isolation of Staphylococci (Schleifer and Krämer, 1980)

| Basal medium: |  |
|--------------|---|
| Tryptone or peptone from casein | 10.0 g |
| Beef extract | 5.0 g |
| Yeast extract | 3.0 g |
| Glycerol | 10.0 g |
| Sodium pyruvate | 10.0 g |
| Glycine | 0.5 g |
| KSCN | 2.25 g |
| NaH₂PO₄ · H₂O | 0.6 g |
| Na₂HPO₄ · 2H₂O | 0.9 g |
| LiCl | 2.0 g |
| Agar | 13.0 g |
| Distilled H₂O | 1 liter |

Adjust pH to 7.2. Autoclave at 121°C for 15 min, cool down in water bath to 45°C and add 10 ml of a 0.45% sterile-filtered solution of sodium azide. Mix medium thoroughly and pour immediately into Petri dishes. The medium can be stored at 4°C for at least one week. Staphylococci can be detected in various foods at levels as low as 100 colony-forming-units (CFU)/g of food. As in the case of KRA-NEP agar, the addition of egg yolk or pork plasma to the basal medium can provide a basis for distinguishing S. aureus from the coagulase-negative staphylococci.

The recovery of some animal species (e.g., S. caprae and S. chromogenes) can be improved by adding 5% sheep blood and/or reducing the level of sodium azide in SK agar from 45 to 15 mg/liter (Harvey and Gilmour, 1988).

Isolation of Staphylococci from Clinical Specimens

The isolation and enumeration of staphylococci from clinical specimens are routine operations in the hospital and veterinary clinical laboratory. Procedures for handling specimens and isolating and enumerating staphylococci can be found in the following texts: American Society for Micro-
biology (ASM) Manual of Clinical Microbiology, seventh edition (Murray et al., 1999), A Guide to Specimen Management in Clinical Microbiology, second edition (Miller, 1998) and Clinical Microbiology Procedures Handbook (Isenberg, 1994).

Staphylococci from a variety of clinical specimens are usually isolated in primary culture on blood agar and in a fluid medium such as thioglycolate broth. A general discussion of the preparation of specimens for primary culturing and inoculation of media and colony isolation methodology can be found in the ASM Manual of Clinical Microbiology (Murray et al., 1999).

Isolation and Culture from Clinical Specimens (Kloos and Bannerman, 1999)

Every specimen should be plated onto blood agar (preferably sheep blood agar) and other media as indicated. On blood agar, abundant growth of most staphylococcal species occurs within 18–24 h. Since most species cannot be distinguished from one another during this time period, colonies should be picked at this time only for preliminary identification testing (e.g., when specimens are taken from patients with acute infections). Colonies should be allowed to grow for at least an additional two to three days before the primary isolation plate is confirmed for species or strain composition (Kloos and Schleifer, 1975b; Kloos and Schleifer, 1975c). This growth period is particularly important when the sampling of more than one colony is needed to obtain sufficient inocula for determining the predominant organism or to obtain a pure culture. Failure to hold plates for 72 h, can result in 1) selection of more than one species or strain if sampling yields an inoculum of two or more colonies, 2) selection of an organism(s) not producing the infection, if the specimen contains two or more different species or strains, and 3) incorrectly labeling a mixed culture as a pure culture. Colonies should be Gram-stained, subcultured, and tested for genus, species, and strain properties. It should be noted that most staphylococci of major medical interest produce growth in the upper as well as the lower anaerobic portions of thioglycolate broth or semisolid agar (Kloos and Schleifer, 1975c).

Fecal specimens suspected of containing infecting staphylococci (e.g., associated with staphylococcal enterocolitis) and other specimens from potentially heavily contaminated sources should also be inoculated on a selective medium such as SK agar (described above). Columbia CNA agar, lipase-salt-mannitol agar (LSM; Remel, Lenexa, KS, USA), tellurite glycine agar, phenylethyl alcohol agar, or mannitol salt agar. These media inhibit the growth of Gram-negative bacteria in addition to some other contaminating species. Incubation of these cultures should be for at least 48–72 h for discernible colony development.

Blood Cultures and Catheter Tips

Blood cultures are usually indicated when there is a sudden increase in the pulse rate and temperature of the patient, a change in sensorium, and the onset of chills, prostration, and hypotension. Timing of collection is usually not critical when bacteremia is expected to be continuous; however, bacteremia often is intermittent. In these cases, timing may be very important, and bacteremia may precede the onset of fever or chill by as much as 1 h. Staphylococci are one of the major groups of bacteria that can produce a serious bacteremia.

Ideally, blood specimens should be collected before administration of antimicrobial agents and as close as possible to a fever spike. Blood for culture should be collected aseptically, first by cleaning the venipuncture site with 70% alcohol and then with an iodine preparation in a concentric fashion. The iodine should be allowed to dry. The venipuncture site should not be touched after cleansing. After venipuncture, the iodine should be removed from the skin with alcohol. It is recommended that 10–20 ml/set be collected in adults and 1–10 ml/set in infants. Also for acute sepsis, 2 or 3 sets should be collected from separate sites all within 10 min; for acute endocarditis, 3 sets should be collected from separate sites within 1–2 h; and for subacute endocarditis, 3 sets should be collected from separate sites taken 15 min apart. Initial processing of a blood specimen depends on the culture system used. All bacterial blood cultures should be incubated at 35°C for up to 7 days for evidence of growth. Gram-stained smears and subcultures of suspected positive cultures should be prepared immediately.

Culture of catheter tips may be performed to determine the source of bacteremia. The most commonly used method is the semi-quantitative method (Maki et al., 1977). A 5-cm segment of the distal tip is rolled across a blood agar plate four times. Cultures yielding > 15 bacterial colonies are considered clinically significant for a catheter-related infection.

Preparation of Body Fluid Cultures

Staphylococci may infect a variety of body fluids in addition to blood, such as cerebrospinal fluid (CSF) and joint, intraocular, pericardial, peritoneal, and pleural space fluids. It usually is easiest to establish the etiological agent in infections of normally sterile body sites, provided puncture and handling of the specimen are performed under conditions of strict asepsis. The skin
should be disinfected with a 2% solution of tincture of iodine. The specimen should be injected immediately into a sterile (screw-cap) tube or bottle. The specimen should be transported immediately to the laboratory for testing. Since only a small number of microorganisms may be present in clear or slightly cloudy fluids, volumes > 1 ml should be centrifuged to concentrate the organisms. A portion of the sediment is used for Gram staining and to inoculate the media.

**Preparation of Urine Cultures**  
Staphylococal urinary tract infections in humans are commonly caused by *S. saprophyticus, S. epidermidis* and *S. aureus*. Acceptable methods for urine collection include midstream clean catch, catheterization, and suprapubic aspiration. At least 1 ml of midstream urine should be collected in a sterile, wide-mouth container. Unpreserved specimens should be cultured within 2 h of collection or stored in a refrigerator for no more than 24 h. Most references to diagnostic criteria state that a significant bacteriuria occurs when there are 100,000 cells or more per ml in a clean-voided, midstream specimen obtained from asymptomatic patients. With acute dysuria and frequency in young, sexually active females, a colony count as low as 100 per ml may be a useful criterion. Many significant urinary tract infections due to *S. saprophyticus* are associated with only 100 to 10,000 colonies per ml. If significant, these low counts may be substantiated by repetition of the procedure. Significant bacteriuria may also be determined by microscopic examination of a Gram-stained smear of uncentrifuged urine. The presence of at least two bacteria per 1000-X microscopic field of the Gram-stained smear is approximately equal to 100,000 or more cells per ml (Pollock, 1983).

**Isolation of Staphylococci from Skin and Mucous Membranes**  
Several basic methods are available for isolating staphylococci and other aerobic bacteria from skin and the adjacent mucous membranes (reviewed by Noble and Somerville, 1974). Washing or swabbing methods disperse cutaneous bacteria to provide samples of uniform composition. They break up large aggregates or microcolonies on skin into smaller colony-forming units (CFU) and in some cases single cells. Impression methods estimate the number of microcolonies or aggregates of bacteria on the skin surface. Biopsy methods can determine the location of bacteria in microniches on skin. Most of the sampling of aerobic bacteria on skin and mucous membranes has been performed using scrubbing and swabbing methods. The swab technique described by Kloos and Musselwhite (1975a) is suitable for use with human as well as other mammalian skin. The medium most widely used for the isolation and culture of natural populations of staphylococci is P agar (Kloos et al., 1974).

**P Agar**  
- Peptone: 10.0 g  
- Yeast extract: 5.0 g  
- Sodium chloride: 5.0 g  
- Glucose: 1.0 g  
- Agar: 15.0 g  
- Distilled water: 1 liter

Adjust pH to 7.5 before autoclaving at 121°C for 15 min.

From each inoculated swab, a series of dilutions are prepared and plated on standard size (15 × 100 mm) P agar plates, in an attempt to obtain 50–300 isolated colonies on a plate for identification and enumeration. Inoculated plates are incubated at 34–35°C for 3–4 days and then held at room temperature for an additional 2 days. Each colony type is enumerated and one or two representatives of each type per plate are examined further for distinguishing genus, species, and strain characteristics. Colony morphology can be a useful supplementary character in the identification of species and strains. Selective media may be used in addition to the nonselective P agar if bacterial and/or fungal populations are very large (e.g., from the human inguinal and perineal area or from the skin of certain animals) and/or if the cutaneous flora contains species producing large, spreading colonies. Pigment production of colonies may be enhanced by the addition of milk, fat, glycerol monoacetate, or soaps to P agar or heart infusion agar (Willis et al., 1966).

**Isolation of Staphylococci from Water**  
The presence of potentially pathogenic staphylococci in recreational waters, swimming pools, water that might be added to foods, and hydrotherapy pools poses a threat to human health (reviewed by Evans, 1977). Most attention is focused on the presence in water of *S. aureus* although several other staphylococcal species are also opportunistic pathogens. Staphylococci are somewhat resistant to halogen disinfectants. For this reason, significant numbers of these organisms can remain viable for extended periods of time in inadequately treated bathing places. Methods for the recovery and enumeration of pathogens from water are described in the manual jointly published by the American Public Health Association (APHA) and Water Environment Federation entitled *Standard Methods for the Examination of Water and Wastewater*, twentieth edition (Clesceri et al., 1998).
Phylogeny

On the basis of comparative 16S rRNA sequence studies, the genera *Staphylococcus* and *Macrococcus* belong to the Gram-positive bacteria with a low DNA G+C content. They are closely related to bacilli and other Gram-positive bacteria with low DNA G+C content such as enterococci, streptococci, lactobacilli and listeria (Fig. 1). Combining *Staphylococcus*, *Gemella*, *Macrococcus* and *Salinicoccus* within the family Staphylococcaceae has been proposed (Garrity and Holt, 2001).

The genera *Staphylococcus* and *Macrococcus* are monophyletic (Fig. 2) and well separated from each other with intergenera 16S rRNA sequence similarities of 93.4–95.3%. The intragenus similarities are significantly higher with at least 96.5% for staphylococci and 97.7% for macrococi. On the basis of DNA-DNA hybridization studies, staphylococcal species can be grouped (Kloos et al., 1992). The most important ones are the two coagulase-negative and novobiocin-susceptible species groups *S. epidermidis* (e.g., *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saccharolyticus* and *S. warneri*) and *S. simulans* (e.g., *S. carnosos* and *S. simulans*); the two coagulase-negative and novobiocin-resistant species groups *S. saprophyticus* (e.g., *S. cohnii*, *S. saprophyticus* and *S. xylosus*) and *S. sciuri* (e.g., *S. lentus*, *S. sciuri* and *S. vitulus*), and the two coagulase-positive and novobiocin-susceptible species groups *S. intermedius* (e.g., *S. delphini* and *S. intermedius*) and *S. aureus* (e.g., *S. aureus* and *S. aureus* subsp. *anaerobius*).

On the basis of DNA-DNA hybridization studies, the species *M. equiperdicus*, *M. bovicus* and *M. caruselicus* were more closely related to one another than to *M. caseolytics* (Kloos et al., 1998a). Both DNA-DNA hybridization studies and 16S rRNA sequence analysis indicate a closer relationship of the genus *Macrococcus* to the *S. sciuri* species group than to other staphylococcal species.

Identification

General Properties

Members of the genus *Staphylococcus* are Gram-positive cocci (0.5–1.5 µm in diameter) that occur singly, in pairs, tetrads, short chains (three or four cells), and irregular grape-like clusters. They are nonmotile, nonsporeforming, and usually are unencapsulated or have limited capsule formation. Most species are facultative anaerobes and are positive for the catalase and benzidine tests. With the exception of *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, growth is more rapid and abundant under aerobic conditions. These exceptional staphylococci are also catalase-negative. Most species contain *a*- and *b*-type cytochromes. The exceptional species *S. lentus*, *S. sciuri* and *S. vitulus* contain *a*-, *b*- and *c*-type cytochromes. Menaquinones are unsaturated (normal). The G+C content of DNA is in the range of 30–39 mol%. The genome size is in the range of 2000–3000 kb (George and Kloos, 1994; Kloos et al., 1998b). Staphylococci are generally susceptible to lysostaphin (some species more than others), furazolidone and nitrofuran, and resistant to erythromycin and bacitracin at low levels. In the laboratory routine, rapid distinction of staphylococci from micrococci can be made by demonstrating the susceptibility of staphylococci to 200 µg of lysostaphin per ml and resistance to erythromycin at 0.04 µg per ml, plus the production of acid from glycerol (Schleifer and Kloos, 1975b) or, alternatively, demonstrating susceptibility of staphylococci to a 100 µg furazolidone disk and resistance to a 0.04-unit bacitracin disk (Baker, 1984). Furthermore, staphylococci, with the exceptions of *S. lentus*, *S. sciuri* and *S. vitulus* exhibit a negative reaction with the rapid modified oxidase test, whereas micrococci are positive for this test (Fallier and Schleifer, 1981).
Members of the genus *Macrococcus* are Gram-positive cocci (1.1–2.5 μm in diameter) that occur mostly in pairs and tetrads, and occasionally single and arranged in short chains (Kloos et al., 1998a). They are nonmotile, non-sporforming, and usually are unencapsulated. Macrococci are marginally facultative anaerobes; growth occurs better under aerobic conditions. They are positive for catalase and oxidase activities. They are resistant to bacitracin and lysozyme (25 μg/ml), and susceptible to furazolidone. Macrococci can contain α-, β- and/or c-type cytochromes. The G+C content of DNA is in the range of 38–45 mol%. The genome size is in the range of 1500–1800 kb.

Cell Wall Composition

The ultrastructure and chemical composition of the cell wall of staphylococci is similar to that of other Gram-positive bacteria. It consists of a thick (usually 60–80 nm), rather homogeneous, and not very electron-dense layer. It is made up of peptidoglycan, teichoic acid, and protein (Schleifer, 1983). Macrococci, with the possible...
exception of *M. caseolyticus*, do not have detectable levels of teichoic acids.

A characteristic feature of the peptidoglycan of staphylococci is the occurrence of glycine-rich interpeptide bridges. Penta- and hexaglycine interpeptide bridges are found in about half of the staphylococcal species (peptidoglycan type: Lys-Gly$_{5,6}$). In most of the other half, a minor part of the glycine residues can be replaced with L-serine (peptidoglycan type: Lys-Gly$_{4}$, Ser). *Staphylococcus sciuri*, *S. lentus*, *S. fleuretii* and *S. vitulus* may have an L-alanine instead of a glycine residue bound to lysine of the peptide subunit (peptidoglycan type: Lys-Ala-Gly$_{2}$). The peptidoglycan types for macrococci can be either Lys-Gly$_{3,4}$, L-Ser (*M. caseolyticus*, *M. equiperdicus* and *M. carouselicus*) or Lys-Gly$_{3}$, L-Ser (*M. bovicus*).

Staphylococcal cell wall teichoic acids are water-soluble polymers containing repeating phosphodiester groups that are covalently linked to peptidoglycan. They consist of polyol (glycerol and ribitol) sugars and/or N-acetylamino sugars. Most staphylococci contain glycerol or ribitol teichoic acids. The teichoic acids consist of polymerized polyol phosphates that are substituted with various combinations of sugars and/or N-acetylaminosugar residues, and also ester-linked D-alanine residues. In some cases, N-acetylamino sugar residues can also form an integral part of the polymer chain (Endl et al., 1983; Endl et al., 1984). The occurrence of the same major components does not always mean that the structure of teichoic acid is identical; for example, the teichoic acids of *S. capitis* and *S. hyicus* show a similar composition but their structures are quite different.

### Differentiation of Species and Subspecies

The classification of species and subspecies of staphylococci and macrococci can be based on a variety of phenotypic character analyses and DNA-DNA (genomic) relationships. In addition, rRNA analysis and ribotyping may be used to describe the relationship of reference and new species. DNA similarity (> 70%) is the criterion that has been used to determine species boundaries in the formal classification of staphylococcal species and most subspecies. Selected phenotypic characters are also useful in classification because they have a high predictive value in identifying DNA similarity groups. Recently, recommended minimal standards for the description of new staphylococcal species have been published (Freney et al., 1988). Characters studied at the cellular and population levels, including morphological and physiological properties, enzyme reactions, and intrinsic resistance to certain antibiotics, have been included in practical identification schemes as shown in Tables 2–5. Molecular studies of phenotypic characters have also provided a basis for determining epigenetic relationships.

#### Table 2. Differentiation of coagulase/clumping factor-positive *Staphylococcus* species and subspecies.

| Character          | *S. aureus* | *S. aureus* subsp. *aureofaciens* | *S. albus* | *S. hyicus* | *S. intermedius* | *S. lugdunensis* | *S. lutreus* | *S. sciuri* subsp. *canicarica* | *S. sciuri* subsp. *rodalionum* | *S. schleiferi* | *S. schleiferi* subsp. *coagulans* |
|--------------------|-------------|----------------------------------|-------------|-------------|------------------|-----------------|-------------|---------------------------------|-------------------------------|----------------|----------------------------------|
| Colony size ±6mm   | +           | +                                | +           | +           | +                | d               | d           | +                              |                                | d              | +                                |
| Colony pigment     | +           | +                                | +           | +           | +                | d               | d           | +                              |                                | d              | +                                |
| Anaerobic growth   | +           | (+)                              | +           | (+)         | +                | +               | d           | (+)                            |                                | d              | +                                |
| Aerobic growth     | +           | ()                               | +           | +           | +                | +               | +           | +                              |                                | d              | +                                |
| Staphylococulase   | +           | +                                | +           | d           | +                | -               | -           | +                              |                                | -              | +                                |
| Clumping factor    | +           | -                                | +           | d           | +                | +               | +           | +                              |                                | d              | +                                |
| Thermonuclease     | +           | +                                | +           | +           | +                | +               | +           | +                              |                                | d              | +                                |
| Hemolysis          | +           | +                                | +           | +           | +                | +               | +           | +                              |                                | d              | +                                |
| Catalase           | +           | -                                | +           | +           | +                | +               | +           | +                              |                                | d              | +                                |
| Modified oxidase   | +           | -                                | +           | +           | +                | +               | +           | +                              |                                | d              | +                                |
| Alkaline phosphatase| +           | +                                | +           | +           | +                | +               | +           | +                              |                                | +              | +                                |
| Pyrrolidonyl arylamidase | -    | ND                               | -           | +           | +                | ND              | -           | +                              |                                | -              | +                                |
| Ornithine decarboxylase | -    | ND                               | -           | +           | ND               | -               | -           | -                              |                                | ND             | ND                               |
| Urease             | d           | ND                               | +           | d           | d                | +               | -           | -                              |                                | -              | +                                |

**Conventional Methods** Conventional methods for the determination of phenotypic characters at the cellular and population levels were developed first and then examined for their correlation to DNA relatedness (reviewed by Kloos...
Table 2. Continued

| Character | S. aureus | S. aureus subsp. an aerogenes | S. delphini | S. pyogenes | S. intermedius | S. lugdunensis | S. warneri | S. sciuri subsp. carnosulus | S. sciuri subsp. rodeonius | S. schleiferi | S. schleiferi subsp. coagulans |
|-----------|-----------|-----------------------------|-------------|-------------|----------------|----------------|-----------|----------------------------|---------------------------|-------------|--------------------------------|
| β-Glucosidase | + | – | – | ND | d | d | + | ND | + | + | – | ND |
| β-Glucuronidase | – | – | ND | + | – | – | ND | – | – | – | ND | – |
| β-Galactosidase | – | – | ND | – | – | – | + | – | – | – | – | (+) ND |
| Arginase d | – | – | ND | d | – | d | – | – | – | – | – | ND |
| Acetate dehydrogenase | – | – | ND | + | + | d | – | – | – | – | – | ND |
| Nitrate reduction | – | – | + | + | + | + | + | + | + | + | + | ND |
| Esculin hydrolysis | – | – | ND | – | – | – | ND | + | + | – | ND | – |
| Novobiocin resistance | – | – | – | – | – | – | – | + | + | – | – | – |
| Acid (aerobically) from: | | | | | | | | | | | | |
| D-Trehalose | + | – | – | + | + | + | + | + | (+) | d | – |
| D-Mannitol | + | ND | (+) | – | (d) | – | d | + | + | – | d |
| D-Mannose | + | – | – | – | + | + | + | + | (d) | (+) | + |
| D-Turanose | + | ND | ND | – | d | (d) | ND | ND | ND | – | ND |
| D-Xylose | – | – | – | – | – | – | + | (d) | – | – | – |
| D-Cellobiose | – | – | ND | – | – | – | ND | (d) | d | – | – |
| L-Arabinitol | – | – | – | – | – | – | – | (d) | – | – | – |
| Sucrose | – | – | – | – | – | – | – | – | – | – | – |
| Raffinose | – | – | ND | – | – | – | – | – | – | – | – |

Symbols: +, 90% or more strains; –, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.

Adapted from Kloo and Bannerman (1999); Kloo and Scheifer (1986); Schleifer (1986).

Table 3. Differentiation of coagulase-negative, novobiocin-susceptible Staphylococcus species and subspecies.

| Character | S. aureus | S. capitis | S. capitis subsp. ureolyticus | S. carnosus | S. carnosus subsp. natalensis | S. sciuri | S. sciuri subsp. intermedius | S. sciuri subsp. hominis | S. sciuri subsp. ureolyticus | S. sciuri subsp. rodeonius | S. sciuri subsp. carnosulus |
|-----------|-----------|------------|-----------------------------|-------------|-----------------------------|-----------|-----------------------------|--------------------------|-----------------------------|---------------------------|-----------------------------|
| Colony size ≤6mm | – | – | – | d | + | + | ND | – | + | + | + | ND |
| Colony pigmem | – | – | – | – | – | – | – | – | – | – | – | – |
| Anaerobic growth | (+) | (+) | (+) | (+) | + | + | + | + | + | + | (d) | (+) |
| Aerobic growth | (+) | + | + | + | + | + | + | + | + | + | + | + |
| Staphylococcalase | – | – | – | – | – | – | – | – | – | – | – | ND |
| Clumping factor | – | – | – | – | – | – | – | – | – | – | (+) | – |
| Thermonuclease | – | – | – | – | – | – | – | – | – | – | – | ND |
| Hemolysis | – | (d) | (d) | (d) | ND | ND | (d) | (+) | – | (+) | (+) | (d) |
| Catalase | + | + | + | + | + | + | + | + | + | + | + | + |
| Modified oxidase | – | – | – | – | – | – | – | – | – | – | – | – |
| Alkaline phosphatase | – | – | – | (+) | + | + | + | + | + | + | + | + |
| Pyruvate dehydrogenase | d | (d) | d | + | ND | d | ND | – | + | + | ND | – |
| Ornithine decarboxylase | – | – | – | ND | ND | (d) | ND | – | + | + | ND | – |
| Urease | – | + | + | + | + | + | + | + | + | + | ND | – |
| β-Glucosidase | – | – | – | ND | ND | (d) | – | d | – | + | ND | + |
| β-Glucuronidase | – | – | – | ND | ND | (d) | – | d | – | + | ND | + |
| β-Galactosidase | (d) | – | – | – | – | – | – | – | – | – | – | ND |
| Arginine dehydrolase | d | d | + | + | + | + | d | + | d | – | d | + |
| Acetoin production | – | d | d | + | + | ND | ND | – | + | d | – | ND | – |

Adapted from Kloo and Bannerman (1999); Kloo and Scheifer (1986); Schleifer (1986).
Table 4. Differentiation of novobiocin-resistant *Staphylococcus* species and subspecies.

| Character          | *S. aureus* | *S. capitis* | *S. capitis* subspecies urealyticus | *S. caprae* | *S. carnosus* | *S. carnosus* subspecies subsp. carnosus | *S. chromogenes* | *S. colensoi* | *S. cohnii* | *S. epidermidis* | *S. equorum* | *S. fallax* | *S. hominis* | *S. hominis* subspecies novobiopticus | *S. intermedius* | *S. kloosii* | *S. mallotus* | *S. simulans* | *S. simulans* subspecies carniecarius | *S. simulans* subspecies rodenticum | *S. xylosus* | *S. warneri* |
|--------------------|-------------|-------------|-----------------------------------|-------------|---------------|-----------------------------------------|---------------|-------------|------------|-----------------|-------------|-----------|-------------|------------------------------------|----------------|------------|-------------|------------|-----------------------------|-------------------|------------|-------------|
| Colony size ±6mm   | d           | d           | +                                  | d           | d             | +                                      | +             | d           | d          | +               | +           | +         | d           | d                                   | d             | +          | d           | +          | +                           | +                 | +          | d           |                |
| Colony pigment     | +           | d           | d                                  | d           | d             | d                                      | d             | d           | d          | +               | +           | +         | d           | d                                   | d             | +          | d           | +          | +                           | +                 | +          | d           |                |
| Anaerobic growth   | –           | –           | +                                  | (+)         | +             | –                                      | d             | d           | d          | d               | +           | +         | d           | (+)                                 | (+)            | (+)        | d           | +          | +                           | +                 | +          | d           |                |
| Aerobic growth     | +           | +           | +                                  | (+)         | +             | +                                      | (+)           | +           | +          | +               | +           | +         | d           | d                                   | d             | d          | d           | +          | +                           | +                 | +          | d           |                |
| Staphylocoagulase  | –           | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Clumping factor    | –           | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Thermonuclease     | –           | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Hemolysis          | –           | (d)         | (d)                                | (d)         | (d)           | (d)                                    | d             | d           | d          | d               | +           | +         | d           | d                                   | d             | d          | d           | d          | d                           | +                 | d          | d           |                |
| Catalase           | +           | +           | +                                  | +           | +             | +                                      | +             | +           | +          | +               | +           | +         | d           | d                                   | d             | +          | d           | +          | d                           | d                 | +          | d           |                |
| Modified oxidase   | –           | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Alkaline phosphatase| (+)         | (+)         | (+)                                | d           | (+)           | d                                      | d             | d           | d          | d               | +           | +         | d           | d                                   | d             | +          | d           | +          | d                           | d                 | +          | d           |                |
| Pyrrolidonyl arylamidase | –       | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Ornithine decarboxylase | –       | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Urease             | –           | –           | +                                  | +           | +             | –                                      | –             | +           | –          | –               | +           | +         | d           | d                                   | d             | d          | d           | +          | d                           | d                 | d          | d           |                |
| β-Glucosidase      | ND          | ND          | ND                                 | ND          | ND            | d                                      | +             | d           | d          | +               | +           | +         | d           | d                                   | d             | d          | d           | +          | d                           | d                 | d          | d           |                |
| β-Gluconoridase    | +           | +           | +                                  | –           | –             | d                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| β-Galactosidase    | d           | +           | +                                  | –           | –             | d                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Arginine dihydrolyase| –         | –           | –                                  | –           | –             | –                                      | –             | –           | –          | –               | –           | –         | –           | –                                   | –             | –          | –           | –          | –                           | –                 | –          | –           |                |
| Acetoin production | –           | d           | d                                  | –           | d             | d                                      | +             | d           | d          | +               | +           | +         | d           | d                                   | d             | d          | d           | +          | d                           | +                 | d          | d           |                |
| Nitrate reduction   | –           | –           | +                                  | +           | +             | d                                      | –             | +           | +          | +               | +           | +         | d           | d                                   | d             | +          | d           | +          | d                           | +                 | d          | d           |                |
| Esculin hydrolysis  | –           | –           | d                                  | –           | –             | d                                      | +             | +           | d          | +               | +           | +         | d           | d                                   | d             | +          | d           | +          | d                           | +                 | d          | d           |                |

Symbols: +, 90% or more strains; −, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.
Adapted from Kloos and Bannerman (1999); Kloos and Scheifer (1986); Schleifer (1986).
Table 4. Continued

| Character          | S. arletae | S. cohni | S. cohni subsp. urealyticus | S. equorum | S. fleurentii | S. gallinarum | S. hominis subsp. novobiocinicus | S. kloosii | S. lentus | S. saprophyticus subsp. bovis | S. sciruri subsp. sciruri | S. sciruri subsp. carlaniiclus | S. sciruri subsp. rodentium | S. terrae | S. vitulina |
|-------------------|------------|----------|------------------------------|------------|---------------|--------------|---------------------------------|------------|----------|-------------------------------|--------------------------|-----------------------------|-----------------------------|----------|-----------|
| Acid (aerobically) from: |            |          |                              |            |               |              |                                 |            |          |                               |                          |                             |                             |          |           |
| D-Trehalose       | +          | +        | +                            | +          | +             | +            | +                               | +          | +        | +                            | +                        |                             |                             | +        | +         |
| D-Mannitol        | +          | d        | +                            | +          | ND            | +            | +                               | +          | +        | +                            | ND                       | ND                          | ND                          | +        | +         |
| D-Mannose         | +          | (d)      | +                            | +          | +             | +            | +                               | +          | (+)      | (d)                          | (d)                      | (+)                         | (+)                         | +        | (d)       |
| D-Turanose        | +          | –        | –                            | d          | +             | ND           | (d)                             | (d)        | ND       | ND                           | ND                       | ND                          | d              | (d)      | (d)       |
| D-Xylose          | +          | –        | –                            | +          | d             | –            | (d)                             | (d)        | ND       | ND                           | d                        | ND                          | ND                          | +        | +         |
| D-Cellobiose      | –          | –        | (d)                          | –          | +             | –            | +                               | –          | +        | +                            | +                        | +                           | +                           | –        | –         |
| L-Arabinose       | +          | (d)      | (+)                          | (d)        | d             | d            | d                               | d          | (d)      | d                            | (d)                      | (d)                         | d                           | –        | –         |
| Maltose           | +          | (d)      | (+)                          | d          | +             | d            | d                               | (d)        | +        | +                            | +                        | +                           | +                           | –        | –         |
| Sucrose           | +          | –        | –                            | +          | +             | +            | +                               | +          | +        | +                            | (+)                      | (+)                         | (+)                         | +        | +         |
| N-Acetylglucosamine | –        | d       | ND                           | –          | –             | –            | –                               | –          | –        | –                            | –                        | –                           | –                           | –        | –         |
| Raffinose         | –          | –        | –                            | –          | –             | –            | –                               | –          | –        | –                            | –                        | –                           | –                           | –        | –         |

Symbols: +, 90% or more strains; –, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. (d) indicate a delayed reaction.

Adapted from Kloos and Bannerman (1999); Kloos and Scheifer (1986); Schleifer (1986).

Table 5. Conventional identification methods useful for the differentiation of *Macrococcus* species.

| Character          | M. caroleicus | M. equipericicus | M. bovis | M. caroleicus | M. caroleicus | M. sciruri subsp. sciruri | M. sciruri subsp. arcanlua | M. sciruri subsp. rodentium | M. terrae | M. fleureti | M. vitulina |
|-------------------|---------------|-----------------|----------|---------------|---------------|----------------------------|---------------------------|-----------------------------|-----------|-------------|------------|
| Anaerobic growth  | d             | –               | –        | –             | (d)           | (d)                       | (d)                       | (d)                         | –         | –           | –          |
| Heat-stable nuclease | d             | –               | d        | +             | –             | –                         | –                         | –                           | –         | –           | –          |
| Alkaline phosphatase | –             | –               | d        | +             | –             | –                         | –                         | –                           | –         | –           | –          |
| Pyrrolidonyl arylamidase | +             | –               | –        | +             | d             | d                         | (d)                       | (d)                         | –         | –           | –          |
| Urease            | –             | d               | d        | –             | –             | –                         | –                         | –                           | –         | –           | –          |
| β-Glucosidase     | –             | –               | d        | –             | +             | +                         | +                         | ND                          | d         | –           | –          |
| Acetoin production | +             | –               | –        | –             | –             | –                         | –                         | –                           | –         | d           | –          |
| Nitrate reduction | +             | –               | –        | –             | +             | +                         | +                         | +                           | +         | +           | +          |
| Esulin hydrolysis  | d             | d               | –        | +             | +             | +                         | +                         | d                           | –         | –           | –          |
| Acid (aerobically) from: |               |                 |          |               |               |                           |                           |                             |           |             |            |
| D-Mannitol        | –             | +               | d        | +             | +             | +                         | +                         | +                           | ND        | +           | +          |
| D-Mannose         | –             | –               | (d)      | (d)           | (+)           | (+)                       | (+)                       | (+)                          | +         | +           | +          |
| D-Turanose        | –             | –               | –        | (d)           | (d)           | (+)                       | (+)                       | (+)                          | +         | +           | +          |
| D-Xylose          | –             | –               | –        | –             | d             | (d)                       | (d)                       | (d)                          | d         | –           | –          |
| D-Cellobiose      | –             | –               | –        | –             | d             | d                         | d                         | d                           | –         | –           | –          |
| L-Arabinose       | –             | –               | –        | d             | (d)           | (d)                       | (d)                       | (d)                          | –         | –           | –          |
| Maltose           | +             | d               | d        | (d)           | d             | (d)                       | d                         | d                           | –         | –           | –          |
| Sucrose           | +             | d               | d        | (d)           | (d)           | d                         | (d)                       | (d)                          | –         | –           | –          |
| Raffinose         | –             | –               | –        | –             | –             | –                         | –                         | –                           | –         | –           | –          |

Symbols: +, 90% or more of strains positive; –, 90% or more of strains negative; d, 11 to 89% of strains positive; ND, not determined. (d) indicate a delayed reaction.
and Schleifer [1986] and Schleifer [1986]). Key characters now used for species and subspecies identification include the following: colony morphology, oxygen requirements, coagulase, clumping factor, heat-stable nuclease (thermo-nuclease), hemolysins, catalase, oxidase, alkaline phosphatase, urease, ornithine decarboxylase, pyrrolidonyl arylamidase, β-galactosidase, aec-toin production, nitrate reduction, esculin hydrolysis, aerobic acid production from a variety of carbohydrates including D-trehalose, D-mannitol, D-mannose, D-turanose, D-xylene, D-cellobiose, L-arabinose, maltose, α-lactose, sucrose, and raffinose, and intrinsic resistance to novobiocin and polymyxin B (reviewed by Kloos and Bannerman, 1999). Some conventional methods may require up to three to five days before a final result can be obtained, while others only require several hours for interpretation. They are usually quite reliable and have served as a reference for more recent studies aimed at simplifying and expediting character analyses.

**RAPID IDENTIFICATION SYSTEM** To facilitate identification in the routine or clinical laboratory, several manufacturers have developed rapid species identification kits or automated systems requiring only a few hours to one day for the completion of tests. Identification of a number of the *Staphylococcus* species can be made with an accuracy of 70 to > 90% using the commercial systems (Kloos and Bannerman, 1994). Since their introduction, these systems have been improved and expanded to include more species. Their reliability will continue to increase as the result of a growing database and the addition of more discriminating tests. *Staphylococcus aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans* and *S. intermedius* can be identified reliably by most of the commercial systems now available. For some systems, reliability depends upon additional testing as suggested by the manufacturer and/or by published evaluations of the product. Some identification systems now available include the following: RAPIDEC Staph (for identification of *S. aureus*, *S. epidermidis* and *S. saprophyticus*) and API STAPH (bioMérieux Vitek, Inc., Hazelwood, MO, USA); VITEK, a fully automated microbiology system that uses a Gram-positive identification (GPI) card (bioMérieux Vitek); MicroScan Pos ID panel (read manually or on MicroScan instrumentaion) and MicroScan Rapid Pos ID panel (read by the WalkAway systems; in addition, the ID panels are available with antimicrobial agents for susceptibility testing; Dade MicroScan, Inc., West Sacramento, CA, USA); Crystal Gram-Positive Identification System, Crystal Rapid Gram-Positive Identification System, Pasco MIC/ID Gram-Positive Panel, and the Phoenix, an automated identification system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA); GP MicroPlate test panel (read manually, using Biolog MicroLog system, or automatically with the Biolog MicroStation system; Biolog, Hayward, CA, USA); MIDI Sherlock Identification System Microbial Identification System (MIS) that automates microbial identification by combining cellular fatty acid analysis with computerized high-resolution gas chromatography (MIDI, Newark, DE, USA); and Riboprinter Microbial Characterization System (Qualicon, Inc. Wilmington, DE, USA), based on ribotype pattern analysis.

Rapid detection of the species *S. aureus* can be made using the AccuProbe culture identification test for *S. aureus* (Gen-Probe, Inc., San Diego, CA, USA). This test is a DNA probe assay directed against rRNA, and it is very accurate (100% specificity). Tube coagulase-negative and slide test-negative strains of *S. aureus* should be identified correctly by the AccuProbe test. Additionally, PCR analysis of the 16S–23S rRNA intergenic spacer region has preliminarily shown successful results in discriminating among 31 *Staphylococcus* species (Mendoza et al., 1998). PCR analysis allows for the identification of pure culture staphylococci within 24–48 h.

**Physiology**

**Sugar Transport**

Sugar transport, metabolism, and catabolite repression of staphylococci is best studied in the pathogenic species *S. aureus* and the nonpathogenic species *S. xylosus*. Two types of carbohydrate transport have been identified and studied in staphylococci: 1) the phospho(enol)pyruvate (PEP):sugar phosphotransferase system (PTS), which is responsible for the binding, transmembrane transport, and phosphorylation of numerous sugar substrates, and 2) the PTS-independent carbohydrate transport in which the sugar is transported via a permease and is subsequently phosphorylated by an ATP-dependent kinase.

The known sugar transport systems in *S. aureus* and *S. xylosus* are summarized in Table 6. The type of transport used depends on the sugar and staphylococcal species. For some sugars, e.g., glucose, both types of transport systems are present in one species to ensure efficient transport, and they are functional under specific environmental conditions. Studies with PTS-deficient strains of *S. aureus* (Reizer et al., 1988), for example, have demonstrated the utilization of glucose by both PTS-dependent and PTS-independent mechanisms.

**The PHOSPHOTRANSFERASE System (PTS)** The PTS delivers exogenous carbohydrates as phos-
phosphate esters to the cell cytoplasm. It consists of two nonspecific, energy-coupling components, enzyme I (EI, ptsI) and a heat-stable phosphocarrier protein (HPr, ptsH), as well as several sugar-specific multiprotein permeases known as enzymes II (EIIA, B and C). In most cases, enzymes IIA and IIB are located in the cytoplasm, while enzyme IIC forms a membrane channel.

EI and HPr play a central role in the PTS-mediated uptake of most sugars; both enzymes are soluble. The 80-kDa EI of S. aureus has been partially purified and appears to be monomeric. The EI protein is phosphorylated by PEP, a reaction that requires Mg$^{2+}$. The phosphorylation occurs at a histidine residue in the N3 position. HPr is a small protein with a M$_r$ of 8300; its amino acid sequence has been determined (Beyreuther et al., 1977). Also, HPr is phosphorylated by EI-P in position N1 of His-15 (Schrecker et al., 1975). A comparison of the primary structures of HPr proteins of various Gram-positive and -negative bacteria revealed a similar size and three highly conserved centers (Reizer et al., 1988).

### GLUCOSE-SPECIFIC PTS

The data available on staphylococcal PTS-dependent glucose uptake are restricted to S. carnosus. Two genes located next to each other, glcA and glcB, have been cloned; the genes complement an *Escherichia coli* mutant strain deficient in glucose uptake (Christiansen and Hengstenberg, 1996). The GlcA and GlcB proteins are highly similar to each other (69% identity) and to glucose-specific enzyme II proteins from *Bacillus subtilis* and *E. coli*.

The two *S. carnosus* PTS EI permeases have fused EII domains in the order EIICBA. Glucose is the primary substrate of both permeases, but various glucosides may also be recognized (Christiansen and Hengstenberg, 1999). The gene *glcT* is immediately upstream of *glcA* and the deduced amino acid sequence of its protein GlcT shows a high degree of similarity to bacterial regulators involved in antitermination (Stülke et al., 1998). Interestingly, the activity of these regulators is controlled by PTS-mediated phosphorylation. A putative transcriptional terminator partially overlapping an inverted repeat, which could be the target site for the antiterminator protein GlcT, is found in the *glcT-glcA* intergenic region. This organization resembles the *glcT-ptsG* region of *B. subtilis*, encoding the GlcT antiterminator protein and the glucose-specific enzyme II, respectively (Stülke and Hillen, 1999). Therefore, *glcA* expression in *S. carnosus* is most likely controlled by antitermination. Studies of *S. carnosus* GlcT activity in the heterologous host *B. subtilis* have indicated that the protein is indeed able to cause antitermination (Knezevic et al., 2000).

In *S. aureus* as well as in *S. xylosus*, genes encoding glucose-specific PTS permeases are not arranged in tandem. *Staphylococcus carnosus* may therefore be an exception among the staphylococci. As already mentioned, glucose uptake does not solely rely on PTS activity since HPr or EI mutants of *S. aureus* still ferment glucose. The same is also true for *S. xylosus* (Jankovic and Brückner, 2002) and *S. carnosus* (Brückner, 1997); the entry of glucose into the cells by two different mechanisms may thus be a general phenomenon.

### LACTOSE-SPECIFIC PTS

The EIIA$^{lac}$ protein of the lactose-specific PTS of *S. aureus* consists of 103 amino acids (Stüber et al., 1985). Each of the
three subunits is phosphorylated via HPr-P at the N3 position of His-82 (Sobek et al., 1984). The lactose-specific enzyme EIICBac of \textit{S. aureus} couples translocation to phosphorylation of the transported lactose. It is composed of the N-terminal membrane-bound IIC domain, which includes the sugar-binding site, and the C-terminal IIB domain, which contains the phosphorylation site at Cys-476 (Peters et al., 1995). The kinetics of various EIICB fusion constructs have been investigated (Kowolik and Hengstenberg, 1998). The protein, which normally functions as a trimer, is believed to separate into its subunits after phosphorylation. Some of its structural features, like the presence of two histidine residues at the active site, seem to be common to all enzymes, although there is no overall structural similarity to any PTS proteins or to any other proteins in the Protein Data Bank (Sliz et al., 1997). Relevant PTS genes annotated in the genome of \textit{S. aureus} N315 are shown in Table 7.

**CHAPTER 1.2.1**

**The Genera \textit{Staphylococcus} and \textit{Macrococcus}**

Table 7. PTS and non-PTS sugar transporters of \textit{S. aureus} N315 and \textit{S. aureus} NCTC 8325.

| Gene designation | Encoded protein |
|------------------|----------------|
| **General PTS genes** (organized in an operon) |
| \textit{ptsH} | Phosphocarrier protein HPr |
| \textit{psl} | Phosphoenolpyruvate-protein phosphatase, enzyme I |
| **Glucose-specific PTS genes** |
| \textit{ptsG} | Glucose-specific IIABC component |
| \textit{glaA} | Glucose-specific IIA homolog |
| \textit{pbaA} | N-Acetylglucosamine-specific IIABC component |
| \textit{milF} | Mannitol-specific IIBC component |
| \textit{milA} | Mannitol-specific IIA component |
| \textit{lacE} | Lactose-specific IIBC component |
| \textit{lacF} | Lactose-specific IIA component |
| \textit{serA} | Sucrose-specific IIBC component |
| \textit{ptaA} | N-Acetylglucosamine-specific IIABC component |
| \textit{treP} | Trehalose-specific enzyme II |
| **Non-PTS transporters** |
| \textit{gntP} | Gluconate permease |
| \textit{uhpT} | Hexose phosphate transport protein |
| \textit{rbsD} | Ribose permease |
| \textit{glpT} | Glycerol-3-phosphate transporter |

Symbol and abbreviation: PTS, phosphoenolpyruvate (PEP)sugar phosphotransferase system; and –, not yet assigned.

*Genome sequenced by Kuroda et al. (2001).*

*Sequenced at the University of Oklahoma Health Sciences Center.*

also in this and perhaps other staphylococcal species, glucose is taken up by both PTS-dependent and PTS-independent mechanisms.

\textit{Staphylococcus xylosus} is unable to utilize arbutin, raffinose, cellobiose, sorbitol, rhamnose and fucose, and fermentation of salicin and ribose is very weak. Sucrose and fructose are transported exclusively via the PTS system, and PTS plays a central role in trehalose, mannose, maltose, maltotriose, and mannitol transport. Galactose and lactose (Bassias and Brückner, 1998), the pentoses xylose, ribose and arabinose, the corresponding pentitols, and glycerol are clearly non-PTS sugars (Lehmer and Schleifer, 1980; Table 6). The lactose utilization enzymes of \textit{S. xylosus} comprise LacP (lactose permease) and LacH (β-galactosidase). The lacR gene, found upstream of \textit{lacPH} encodes the activator (Bassias and Brückner, 1998). Lactose transport and β-galactosidase activity are induced by the addition of lactose to the growth medium. The
lacPH promoter is also subject to carbon catabolite repression mediated by the catabolite control protein CcpA. Relevant PTS-independent transporter genes annotated in the genome of S. aureus N315 are shown in Table 7.

**The Glycolytic Pathway** Staphylococci are facultatively anaerobic microorganisms. The fructose 1,6-bisphosphate (FBP) and the oxidative pentose phosphate (PP) pathways are the two central routes of glucose metabolism. There is no evidence for the existence of the Entner-Doudoroff pathway. Earlier studies of glucose metabolism, mainly confined to the oxidative aspects in S. aureus, are reviewed by Blumenthal (1972).

The S. aureus N315 genome sequence is available (Kuroda et al., 2001), allowing all genes of the FBP glycolytic pathway to be annotated. The genes are listed in the order of the enzymatic steps of the pathway in Table 8.

**Galactose and Lactose Metabolism (the Leloir and the Tagatose-6-P Pathways)** In many microorganisms, galactose is usually metabolized via the Leloir pathway (Bissett and Anderson, 1973; Bissett and Anderson, 1974). In S. aureus, galactose is converted to D-galactose 6-P, which is further metabolized through tagatose derivatives (Fig. 3). A study of the distribution of the tagatose-6-P pathway in various staphylococcal species has revealed that the key enzyme of this pathway, tagatose-6-P kinase, is found in S. aureus, S. epidermidis and S. hominis. These species do not possess enzymes of the Leloir pathway. The tagatose-6-P kinase is inducible by growth on galactose or lactose. In contrast, S. intermedius, S. saprophyticus and S. xylosus use galactose only via the Leloir pathway and not via the tagatose-6-P pathway (Schleifer et al., 1974).

**Table 8. Genes of the FBP pathway of S. aureus N315 and S. aureus NCTC 8325.**

| Gene designation | Encoded protein                                      |
|------------------|-----------------------------------------------------|
| —                | Similar to glucokinase                               |
| pgi              | Glucose-6-phosphate isomerase A                      |
| pfk              | 6-Phosphofructokinase                               |
| fbaA             | Fructose-bisphosphate aldolase                       |
| tpi              | Triosephosphate isomerase                            |
| gap              | Glyceraldehyde-3-phosphate dehydrogenase             |
| pgk              | Phosphoglycerate kinase                              |
| pgm              | 2,3-Diphosphoglycerate-independent phosphoglycerate mutase |
| eno              | Enolase (2-phosphoglycerate dehydrogenase)           |
| pykA             | Pyruvate kinase                                      |

Symbol and abbreviation: —, not yet assigned; and FBP, fructose 1,6-bisphosphate.

*Genome sequenced by Kuroda et al. (2001).

*Sequenced at the University of Oklahoma Health Sciences Center.

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Fig. 3. Lactose and galactose pathways of staphylococci. Transport of lactose and galactose and their catabolism are shown. In S. aureus, lactose and galactose are transported by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). Internalized lactose 6-phosphate is hydrolyzed by a phospho-β-galactosidase to yield galactose 6-phosphate and glucose. Galactose 6-phosphate is catabolized through the tagatose 6-phosphate pathway. This pathway most likely exists in staphylococci exhibiting lactose PTS activity. In S. xylosus and probably other staphylococcal species that do not possess a lactose PTS, a permease is responsible for the transport of lactose. Galactose uptake has not been studied in these species. Nonphosphorylated lactose is hydrolyzed by a β-galactosidase to yield glucose and galactose. Galactose is likely catabolized through the Leloir pathway. In both staphylococcal species, glucose 6-phosphate, produced by a glucose kinase, enters the FBP pathway, the main glycolytic pathway in staphylococci. Only the encoded products of the galactoside-specific genes are shown. CM, cytoplasmic membrane; EII<sup>lac</sup>, lactose-specific enzyme II; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; and Glc 6-P, glucose 6-P.
et al., 1978). The cloned lactose operon (Fig. 4) of *S. aureus* contains not only the phospho-β-galactosidase gene and genes involved in lactose uptake, but also the genes of the tagatose-6-P pathway (Breidt et al., 1987; Oskouian and Stewart, 1987). The corresponding genes are arranged in a heptacistronic operon (**lacABCD-FEG**). The locus was originally defined by mutations abolishing lactose utilization. The genes **lacFE** code for the galactoside-specific PTS permease, enzyme IIA, and enzyme IIBC (Breidt and Stewart, 1986). The last gene, **lacG**, encodes the phospho-β-galactosidase. The **lacABCD** genes specify the enzymes of the tagatose-6-P pathway, with **lacA** coding for galactose-6-P isomerase, **lacC** for tagatose-6-P kinase, and **lacD** for tagatose-1,6-diP aldolase (Rosey et al., 1991). The gene encoding the lactose operon repressor, **lacR**, is upstream of the **lacABCD-FEG** operon (Oskouian and Stewart, 1990a; Oskouian et al., 1990b). Utilization of lactose and galactose in *S. aureus* relies on the PTS-dependent uptake and phosphorylation of the sugars, resulting in lactose 6-P and galactose 6-P, respectively. Glucose and galactose 6-P are produced from intracellular lactose 6-P by a phospho-β-galactosidase (Hengstenberg et al., 1993). Glucose is metabolized via the FBP pathway, whereas galactose 6-P is degraded via the tagatose-6-P pathway.

Supplementation of the growth medium with lactose or galactose results in the induction of the **lac** genes, with galactose 6-P being the intracellular inducer. Apart from this sugar-specific regulation, the lactose operon of *S. aureus* is also subject to global carbon catabolite repression, but the nature of the regulatory mechanism remains unclear (Oskouian and Stewart, 1990a). It is conceivable that part of the described catabolite repression of the **lac** operon of *S. aureus* is actually caused by glucose-mediated inducer exclusion (Reizer et al., 1989; Saier et al., 1996).

The lactose metabolism pathway described for *S. aureus* is not universal for all staphylococci. In *S. xylosus*, for example, lactose is taken up in the nonphosphorylated form by a lactose permease, a member of the galactoside-pentoside-hexuronide (GPH) cation symporter protein family (Poolman et al., 1996). The lactose permease is encoded by **lacP** and the β-galactosidase by **lacH** (Bassias and Brückner, 1998). After lactose hydrolysis, glucose is phosphorylated by a glucose kinase and catabolized through the FBP pathway, and galactose is likely catabolized through the Leloir pathway (Fig. 3). The genes encoding the enzymes for the Leloir pathway from *S. carnosus* have recently been cloned (B. Krismers, unpublished data), and a gene encoding galactokinase has been isolated from an *S. xylosus* gene library (Brückner et al., 1993). The **lacR** gene of *S. xylosus*, upstream and in the opposite orientation of the **lacPH** operon, encodes an activator belonging to the AraC/XylS family. In contrast to *S. aureus*, *S. xylosus* **lacPH** operon is induced by only lactose, but not galactose, in the culture medium (Bassias and Brückner, 1998).

**Metabolism of Sucrose, Maltose, Mannitol, Ribose and Xylose** The sucrose PTS permease of *S. xylosus*, encoded by **scrA**, is composed of fused EIICB domains (Wagner et al., 1993). The EIIA domain, which is essential for PTS-mediated sucrose uptake, has not been identified. On the basis of sucrose utilization in *E. coli* and *B. subtilis*, it appears questionable whether a separate sucrose-specific EIIA protein exists. In both of these organisms, the EIIA domain specific for glucose, either as an independent enzyme or fused to the IIBC domains of the glucose permease, serves as phosphoryl donor for the sucrose-specific EII enzymes. The GcA and GcB proteins mentioned above would thus be good candidates for sucrose uptake factors.

Internalized sucrose 6-P is cleaved by sucrose-phosphate hydrolyase, the gene product of **scrB**, to yield glucose 6-P and fructose (Brückner et al., 1993). Fructose is subsequently phosphorylated.
by a fructokinase encoded by scrK (R. Brückner, personal communication). The genes scrB and scrK are located next to each other and form an operon. The sucrose permease gene scrA is not located near the scrBK genes. Expression of scrA as well as scrB is induced by sucrose in the medium. Regulation is dependent on the LacI/ GalR-type repressor ScrR, whose gene scrR is found upstream of scrB (Gering and Brückner, 1996). In addition to sucrose-specific ScrR-dependent regulation, the sucrose permease gene scrA is subject to carbon catabolite repression by the catabolite control protein CcpA (R. Brückner, personal communication).

An scr gene cluster, consisting of scrR, scrB and scrK, is also present in S. aureus. It is located next to agrA, the gene encoding the response regulator of the accessory gene regulator system (Agr; Novick et al., 1995). The gene for the sucrose permease has not yet been detected.

Maltose utilization in S. xylosus is dependent on an α-glucosidase (maltsase), whose gene, malA, is found in the malRA operon; malR encodes a regulator belonging to the LacI/GalR family (Egeter and Brückner, 1995). The same enzymatic activity as mediated by MalA in S. xylosus has been characterized in S. aureus, and a gene with high sequence similarity has been identified in the S. aureus genome sequence. Therefore, both staphylococcal species probably use this enzyme to cleave maltose. However, the mechanism of maltose transport remains to be elucidated. In any case, at least one glucose moiety has to be phosphorylated by the glucose kinase for complete maltose catabolism.

The mannitol phosphotransferase system of S. carnosus has been described. The system consists of an EIICB enzyme (encoded by mtlA) and of EIIA (encoded by mtlF), which together form the mannitol-specific PTS permease. The mtlA and mtlF genes are clustered on the S. carnosus chromosome with mtlD, the gene for mannitol-1-phosphate dehydrogenase, which produces fructose-6-P. The gene mtlA is about 2 kb away from mtlF and mtlD (Fischer et al., 1989; Fischer and Hengstenberg, 1992). The nucleotide sequence of this intervening region has not been determined. Staphylococcus aureus possesses an EIIA protein specific for mannitol with the same apparent molecular mass as the S. carnosus enzyme and virtually identical amino acid sequence at the amino terminus and around the phosphorylation site (Reiche et al., 1988). Thus, the mtl systems in the two organisms appear to be very similar.

The uptake of pentoses and pentitols is PTS-independent (Lehmer and Schleifer, 1980). Many staphylococcal species are unable to ferment pentoses, such as D-ribose, L-arabinose, and D-xylose, but are capable of pentose uptake. For example, anaerobically grown S. epidermidis cells are unable to ferment ribose, mannitol, and lactose, but transport measurements confirm that ribose is taken up (Sivakanesan and Dawes, 1980). The genes encoding the enzymes for ribose utilization in S. hyicus have been cloned in S. carnosus, which thereby obtained the ability to utilize D-ribose (Keller et al., 1984). The S. hyicus DNA donor strain possessed D-ribokinase and D-ribose-5-P isomerase, which are absent in S. carnosus. Although the parent S. carnosus strain was unable to utilize ribose, the strain possessed a D-ribose-inducible uptake system that was severely repressed by the addition of glucose.

The xylose degradation genes of S. xylosus are organized in the operon xylABR, consisting of xylR (encoding the Xyl repressor), xylA (encoding xylose isomerase), and xylB (encoding xylose kinase; Sizemore et al., 1991). A xylose transporter is not part of the operon. The XylR protein acts as a repressor that binds to an operator site identified by footprint analysis (Sizemore et al., 1992). An interesting finding is that xylose is the inducer; gel-mobility shift experiments have shown that XylR is inactivated only in the presence of xylose, but not of xylose-P or other phosphorylated sugars. The xyl operon is also subject to glucose catabolite repression at the level of transcription; this control is independent of a functional xylR gene. Indeed, apart from the XylR operator sequence, a catabolite responsive element (cre) is also in front of xylA; cre is involved in glucose repression by binding activated CcpA (Hueck et al., 1994; Egeter and Brückner, 1996).

**Catabolite Regulation**

The major end product of anaerobic glucose metabolism in S. aureus is lactate (73–94%); acetate (4–7%) and traces of pyruvate are also formed (Theodore and Schade, 1965). During aerobic growth, acetate and CO₂ are the predominant end products; only 5–10% of the glucose carbon appears as lactate (Strasters and Winkler, 1963). In the presence of glucose, glycolysis is enhanced, and many enzymes of the pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle are suppressed; furthermore, the oxidation of pyruvate and the cytochrome content are decreased in glucose-grown S. aureus cells. Glucose-mediated catabolite repression is markedly pronounced in staphylococci.

The addition of glucose to aerobically grown S. aureus cells reduces both glucose degradation via the PP pathway and the subsequent oxidation of pyruvate via the TCA cycle. An investigation of the specific activity of various PP enzymes has
revealed no marked differences in extracts of S. aureus grown with or without glucose in nutrient broth. However, the specific activities of two enzymes of the FBP pathway (glyceraldehyde-3-P dehydrogenase and lactate dehydrogenase) are markedly increased in the presence of glucose. Furthermore, in the presence of glucose, the specific activities of the TCA cycle enzymes (succinate dehydrogenase and fumarase) are markedly decreased and fumarase activity is not detectable.

The reduced activities of the TCA cycle enzymes are very likely due to a repression of their biosynthesis; this phenomenon is referred to as the “glucose effect” or “carbon catabolite repression.” The glucose-mediated inhibition of the TCA cycle activity could be triggered by the observed increase in the ATP pool. Citrate synthases of staphylococci are not affected by reduced nicotinamide adenine dinucleotide (NADH), but are severely inhibited by ATP (Hoo et al., 1971). In the presence of glucose, the nicotinamide adenine dinucleotide (NAD)-dependent L-lactate dehydrogenase of S. aureus has considerable activity during aerobic growth; although its activity is about tenfold less than that under anoxic conditions. It is therefore not surprising that in the presence of glucose, some lactic acid is produced even under oxic conditions (Garrard and Lascelles, 1968). During aerobic growth in the absence of glucose, an oxidation of acetate, succinate and malate by resting S. aureus cell suspensions has been observed manometrically (Collins and Lascelles, 1962; Strasters and Winkler, 1963). However, when glucose or galactose (0.04 M) is present in the growth medium, the oxidation of these substrates is abolished. Growth in the presence of glucose also results in a 40-fold decrease in the cytochrome content (Strasters and Winkler, 1963). The presence of a pyruvate dismutation system in staphylococci was first described by Krebs (1937). Pyruvate dehydrogenase activity has been demonstrated in S. aureus and S. epidermidis (Sivakanesan and Dawes, 1980).

Physiological studies with S. epidermidis have shown that the intracellular concentration of FBP influences the LDH activity. During anaerobic growth and in the presence of glucose, a high intracellular concentration of FBP is reached, resulting in maximal LDH activity. In cells grown anaerobically in a glucose-limited medium, the FBP pool is exhausted because of glucose limitation. In this case, LDH is not fully saturated with FBP and its activity is not maximal. Similar results have been obtained with cells grown aerobically in a glucose-excess medium (Götz and Schleifer, 1978).

Class I Aldolase Aldolase is one of the key enzymes of the glycolytic pathway. There are two forms of fructose-1,6-bisphosphate aldolase which can be differentiated by their catalytic and structural properties. Class I aldolases function via the formation of a Schiff base intermediate between the substrate and a lysine amino group of the enzyme. Class II aldolases do not form a Schiff base intermediate, but contain an essential divalent cation, such as Zn$^{2+}$, Ca$^{2+}$ or Fe$^{2+}$, and can be inhibited by EDTA. Since class I aldolases are typical for higher animals and plants and are only found in a few bacteria, it is surprising that nearly all staphylococcal species studied so far possess a class I aldolase (Götz et al., 1980). The only exceptions are S. intermedii and S. hyicus, which possess both classes of aldolases, and S. caseolyticus (reclassified as Macroccocus caseolyticus), which possesses only a class II aldolase (Fischer et al., 1982). The 33-kDa class I aldolase of S. aureus strain ATCC 12600 has been purified; it appears to be active as a monomer (Götz et al., 1980). Later, the S. carnosus TM300 fda gene, encoding the glycolytic fructose-1,6-bisphosphate aldolase (Fda), was cloned in E. coli. The 296-amino-acid protein has a Mr of 32,855. The S. carnosus FDA is also a class I aldolase (EC 4.1.2.13), which does not need divalent metal ions for catalytic activity. Cloning of fda back into S. carnosus led to a sixfold increase of aldolase production and activity. Glucose in the growth medium has a stimulating effect on aldolase production or activity (Witke and Götz, 1993).

**Regulation of Lactate Dehydrogenase Activity** Anaerobically grown S. epidermidis cells ferment glucose with the production of lactate and trace amounts of acetate, formate, and CO$_2$. In S. aureus, glucose is metabolized principally by glycolysis and to a limited extent by the PP oxidative pathway (Sivakanesan and Dawes, 1980). However, certain staphylococcal species, such as S. epidermidis and S. intermedii, are distinguished by a fructose-1,6-bisphosphate (FBP)-activated, NAD-dependent L-lactate dehydrogenase (LDH; Götz and Schleifer, 1975; Götz and Schleifer, 1976). The enzyme has a total Mr of 130,000 and is composed of four subunits.

**Carbon Catabolite Repression** The availability of carbohydrates, especially of glucose, leads to regulatory processes often referred to as the “glucose effect” or “carbon catabolite repression” (Saier et al., 1995; Saier et al., 1996). Numerous publications on S. aureus describe the influence of glucose on a variety of cellular processes, such as utilization of alternative carbon sources, production of extracellular enzymes, activity of glycolytic enzymes, and
cytochrome content. Especially the production of potential virulence factors has attracted considerable attention. However, the mechanism by which glucose exerts its regulatory effect has not been elucidated. The analysis of catabolite repression of inducible systems is quite often complicated by the inability of the inducer to enter the cells in appreciable amounts, when glucose is also present in the medium. In addition to this process, referred to as “inducer exclusion/inducer expulsion,” the rapid removal of internalized inducer, has been described for a number of Gram-positive bacteria (Saier et al., 1996). While inducer exclusion is found in a wide variety of bacteria, inducer expulsion is encountered less frequently. The latter does not appear to operate in S. aureus. The molecular mechanisms leading to inducer exclusion in Gram-positive bacteria are not yet completely understood.

In Gram-positive bacteria, one form of carbon catabolite repression relies on a transcriptional regulator, termed “catabolite control protein A” (CcpA; Henkin, 1996), a member of the GaltR-LacI family of transcription factors (Weickert and Adhya, 1992). CcpA-dependent carbon catabolite repression has been analyzed in some detail in S. xylosus (Egeter and Brückner, 1996). Inactivation of the ccpA gene by insertion of a resistance cassette leads to a pleiotropic loss of transcriptional regulation at several promoters, including the ccpA promoters. This autoregulation of ccpA apparently reflects the need of S. xylosus to balance regulation by CcpA carefully. CcpA shows a relatively weak affinity for its cognate operator sites, termed “catabolite responsive elements” (cre; Hueck et al., 1994), and must, therefore, be activated to bind efficiently to cre. The signal enabling CcpA to bind to DNA is a phosphorylated form of HPr, the phosphocarrier protein of the PTS, which is produced by the HPr kinase (HPrK; Huynh et al., 2000). HPr is phosphorylated at Ser-46 in an ATP-dependent manner and can also be dephosphorylated by the same enzyme. Thus, HPrK constitutes a bifunctional HPr kinase/phosphatase. HPr as well as HPr kinase are absolutely required for CcpA activity in S. xylosus (Jankovic and Brückner, 2002). The genetic organization around the hprK gene of S. xylosus is identical to that in S. epidermidis and S. aureus (Huynh et al., 2000).

In addition to the loss of carbon catabolite repression, the S. xylosus HPr-kinase-deficient strain shows an unexpected phenotype. Its growth is inhibited by glucose. The mutant strain transports glucose at much higher rates than the wildtype strain, and it also produces methyglyoxal, which indicates unbalanced glucose metabolism. Therefore, HPr kinase is not only important to trigger carbon catabolite repression, it is also needed to balance carbohydrate uptake and catabolic capacities of the cell. A summary of the current knowledge of CcpA-dependent catabolite repression and other Gram-positive bacteria with low DNA G+C content is shown in Fig. 5.

Gluconeogenesis
In the absence of a fermentable sugar source, heterotrophic microorganisms normally have to synthesize glucose or glucose 6-P. Pyruvate usually serves as a starting substrate. The pathway is essentially a reversal of glycolysis. Most of the glycolytic enzymes can also catalyze the reverse reaction. In gluconeogenesis, however, three bypass steps are involved: 1) pyruvate to phosphoenolpyruvate, 2) fructose 1,6-bisphosphate to fructose 6-P; and 3) glucose 6-P to glucose. The latter bypass is mostly found in mammals, where glucose has both signal and regulatory functions. Indeed, a glucose-6-phosphatase homolog has not yet been annotated and is very likely not necessary in staphylococci. Enzymes encoded by pycA, pckA and fbp represent the bypass enzymes, while the other enzymes belong to the FBP (fructose 1,6-bisphosphate) pathway and catalyze the reverse reaction.

The postulated gluconeogenesis pathway, according to the genes identified in the S. aureus N315 genome sequence (Kuroda et al., 2001), is shown in Fig. 6.

Oxidative Pentose Phosphate Cycle
The PP cycle is an essential cycle since it serves as the source of pentose phosphates, of which 5-phospho-riboisyl-1-pyrophosphate is required for the biosynthesis of nucleotides. According to the S. aureus N315 genome sequence, the relevant genes are present; only the lactonase gene has not yet been annotated. In Table 9, the corresponding annotated genes are listed.

Aerobic Respiration
TRICARBOXYLIC ACID CYCLE Not much is known about the biochemical properties or enzyme activities of the TCA cycle enzymes in staphylococci under various conditions. Most enzymatic activities were identified, e.g., aconitate (Somerville et al., 2002), isocitrate dehydrogenase, succinate dehydrogenase (Solozhenkin et al., 1991), fumarase, or malate dehydrogenase (Tynecka and Gajdzinska, 1967). In the genome sequence of S. aureus strains N315 and 8325, all the classical TCA cycle genes were annotated (Table 10). One therefore can assume that staphylococci
have in principle a complete TCA cycle; however, bottlenecks exist.

The TCA cycle is fuelled by acetyl CoA and the responsible enzyme is the pyruvate dehydrogenase (PDH), which catalyzes the oxidative decarboxylation of pyruvate, to form acetyl-CoA. The S. aureus PDH complex has been isolated and the last gene of the PDH operon, pdhD, which encodes the lipoamide dehydrogenase (LPD) has been sequenced (Hemila, 1991). The S. aureus genome sequence revealed the E3 core complex subunit and the peripheral subunits E1 and E3 (Table 11). The S. aureus genome contains also a typical pyruvate carboxylase gene that is necessary for initiation of the TCA cycle (Table 6). The activity of this enzyme has been verified.
Fig. 6. Proposed gluconeogenesis pathway in *S. aureus*. PEP, phosphoenol pyruvate; GAP-DH, D-glyceraldehyde-3-phosphate dehydrogenase; and TPI, triose phosphate isomerase.

| Gene designation | Gene function |
|------------------|---------------|
| Pyruvate-DH (E1, E2 and E3 components) | Pyruvate → acetyl-CoA + CO₂ |
| pdhB | Pyruvate dehydrogenase E1 component β-subunit |
| pdhC | Dihydrolipoamide S-acetyltransferase E2 component* |
| | Acetyl-CoA + dihydrolipoamide ↔ CoA + S-acetyldihydrolipoamide |
| pdhD | Dihydrolipoamide dehydrogenase component E3 |
| Pyruvate carboxylase | Pyruvate + HCO₃⁻ + ATP ↔ Oxalacetate + ADP + Pi |
| pycA | Pyruvate carboxylase |

*Hemila (1991). From Kuroda et al. (2001).

**Respiratory Chain** Taber and Morrison (1964) identified three cytochromes in *S. aureus*: cytochrome *a*-602 and cytochromes *b*-555 and *b*-557. The two *b*-type cytochromes have different reactivities. Cytochrome *b*-557 is reduced in the presence of 2-heptyl-4-hydroxyquinoline-N-oxide (HOONO), while all the other hemoproteins of the respiratory chain remain oxidized. In contrast to cytochromes *a*-602 and *b*-555, cytochrome *b*-557 is not reduced by ascorbate-dichlorophenol-indophenol, suggesting that it has a lower reduction potential than the other cytochromes. The most significant difference, however, is that cytochrome *b*-557, unlike cytochromes *b*-555 and *a*-602, does not react with carbon monoxide. Cytochrome *b*-557 appears to be an intermediate electron carrier of the cytochrome *b* or *b₁*-type. Cytochrome *b*-555, which is also found in *S. epidermidis*, binds carbon monoxide and probably is the major terminal oxidase; it is therefore, now referred to as "cytochrome *O"." There are some indications that the prosthetic groups of cytochromes *O* and *b*-557 are protohemin. The role of cytochrome *a*-602 as a terminal oxidase is less clear. It may be involved with nitrate reductase activity. In particular enzyme preparations from *S. aureus*, succinate oxidase, NAD-linked ethanol oxidase, and NADH-oxidase activities are detectable. Succinate oxidase activity is inhibited by ultraviolet (UV) light (340 nm) and surprisingly by amytal (barbiturate A), which inhibits electron transfer from NADH to ubiquinone in higher organisms. The two oxidation-reduction dyes, methylene blue and 2,6-dichlorophenol-indophenol interact with the electron transfer system. The reduced form of 2,6-dichlorophenol-indophenol reduces cytochrome *a*-602 and cytochrome *O* without reducing cytochrome *b*-557. It was found that trimethylamine-N-oxide (TMAO) inhibited the growth of *S. aureus* but not of *S. epidermidis*. This selective inhibition of *S. aureus* was based on the inhibition of the electron transport system by oxidizing the cytochromes in *S. aureus*. It was suggested that the inhibition occurred between cytochrome *b* and cytochrome *O* (Suzuki et al., 1992).

Analysis of cytochrome spectra of *S. aureus* and other staphylococcal species revealed two more minor cytochromes of the *b*-type in addition to cytochromes *a*-602, *b*-557, and *O*-555 (Faller et al., 1980). Cytochrome *b*-552 is found in all staphylococcal species except *S. sciuri*. Cytochromes *b*-560 and *b*-566 are widely distributed in staphylococci. Cytochromes of the *c*-type (e.g., *c*-549 and *c*-554) have been only found so far in *S. sciuri, S. lentus* and *Macrococcus caseolyticus*.

On the basis of the presence of cytochrome *c*, modified oxidase and benzidine tests have been developed which allow rapid differentiation between staphylococci on one hand and micrococci and macrococci on the other hand (Faller and Schleifer, 1981).

Staphylococci possess menaquinones, (MK, vitamin K), as their sole isoprenoid quinones.
There is a species-specific variation with regard to the length of the isoprenoid side chains. The two principal menaquinones have seven to eight isoprene units (Collins and Jones, 1981). Menaquinones are located in the cytoplasmic membrane (White and Frerman, 1967) and play important roles in electron transport and oxidative phosphorylation.

*Staphylococcus aureus* uses aerobically very likely menaquinone oxidase(s) and at least four quinol oxidase-like genes (goxA, B, C and D) were identified. Since the *S. aureus* goxA to goxD genes are very similar to the corresponding *B. subtilis* genes, we expect these genes will have a similar function. As all analyzed staphylococcal species possess a cytochrome a-605, we believe that this cytochrome is part of the *S. aureus* Qox system and functions as a second terminal oxidase. Very likely the ubiquitous cytochrome b-552 (Faller et al., 1980) is associated with cytochrome a-605.

Moreover, the *S. aureus* genome sequences revealed two putative cytochrome d menaquinol oxidases: subunit I homolog (cydA) and subunit II homolog (cydB). Since a d-type cytochrome has never been observed in the staphylococcal cytochrome spectrum (Faller et al., 1980), it is possible that CydAB complex represents the cytochrome O-555 menaquinol oxidase. The very few bits of information on the respiratory components of staphylococci suggest that they have a branched respiratory system consisting of two alternative and menaquinol-dependent terminal oxidases, a cytochrome bo and a cytochrome aa3 oxidase. The still limited knowledge on the overall electron transfer sequence is illustrated in Fig. 7. The scheme is necessarily incomplete since the components of the respiratory chains of *S. aureus* and other staphylococci are not yet fully identified.

The *S. aureus* genome sequence revealed the presence of a typical F$_0$F$_1$-ATP synthase [EC:3.6.3.14] with the following gene order of the various subunits: epsilon (atpC), beta (atpD), gamma (atpG), alpha (atpA), delta (atpH), b (atpF), c (atpE), and a (atpB) subunits.

**Anaerobic Respiration**

In respiration, nitrate is used as an alternative electron acceptor when oxygen is not available. The enzymes for this pathway are only found in bacteria. Two main forms have been described so far; in both, nitrate reduction is coupled to the generation of a proton motive force (p; Boonstra and Konings, 1977; Jones et al., 1980), which is directly utilized as a source of energy or transformed to ATP by a membrane-associated ATPase.

Despite its broad application in food technology, little was known about the biochemistry and genetics of nitrate reduction in *S. carnosus*. A physiological characterization of nitrate and nitrite reduction in *S. carnosus* (Neubauer and Götz, 1996) revealed the presence of a typical dissimilatory nitrate reductase with the following features: 1) repression and inhibition by oxygen, 2) induction by anaerobiosis and nitrate or nitrite, 3) no inhibition by ammonia, 4) energy gain under anaerobic conditions, and 5) localization in the membrane.

Nitrite reductase in *S. carnosus* is a NADH-dependent cytosolic enzyme that forms ammonia as the only end product. The enzyme is regulated similar to dissimilatory enzymes, i.e., it is 1) repressed and inhibited by oxygen, 2) induced by anaerobiosis and nitrate or nitrite, and 3) not inhibited by its end product ammonia. The relatively low energy gain with nitrite under anaerobic conditions points towards a function as terminal electron acceptor, which might be important for the anaerobic recycling of NADH (Neubauer and Götz, 1996). In cells of *S. carnosus*, nitrate is reduced to ammonia in two steps. The first step includes uptake of nitrate, its reduction to nitrite and the subsequent excretion of nitrite. The second step takes place after depletion of nitrate: the accumulated nitrite is imported and reduced to ammonia, which accumulates in the medium. In growth yield studies, nitrate reduction appeared energetically more favorable than nitrite reduction, which makes sense since nitrate reduction is most likely coupled to the generation of a proton motive force. A comparison of the turnover rates in whole cells with glucose as electron donor reveals that nitrate reduction is 10-fold faster than nitrite reduction. For the inhibition of nitrite reduction by nitrate, two possible mechanisms are discussed which are not mutually exclusive. Both are based on the higher activity of nitrate compared to nitrite reductase. Both enzymes compete for NADH, which is in vivo the most important electron

![Fig. 7. Proposed respiratory system in staphylococci.](image-url)
donor. Owing to its higher specific activity, nitrate reductase is expected to perform the bulk of NADH oxidation. In addition, the high rate of nitrate reduction could lead to an internal accumulation of nitrite, which could be the result of a less efficient nitrite reduction or export. High concentrations of nitrite (100 mM) effectively inhibit nitrite reductase but not nitrate reductase activity (Neubauer and Götz, 1996).

**The Nitrate Reductase Operon (narGHJI)**
The enzyme that catalyzes the energy-gaining reduction of nitrate to nitrite is well characterized in *E. coli*. The membrane-bound dissipatory nitrate reductase (NRA) is an enzyme complex composed of three subunits (αβγεσ). The corresponding genes are organized in an operon, *narGHJI* and *narZYW*. The NarJ protein is believed to be involved in the assembly of the native enzyme complex (Dubourdieu and DeMoss, 1992). The γ-subunit (NarV), a b-type cytochrome, receives electrons from the quinone pool. The electrons are then transferred via the iron-sulfur clusters of the β-subunit (NarY) to the molybdenum cofactor bound to the α-subunit (NarG and NarZ, respectively; Blasco et al., 1989).

To gain more insight into the genetic basis of dissimilatory nitrate reduction, a transposon Tn917 mutagenesis in *S. carnosus* TM300 was performed and mutants defective in the conversion of nitrate to nitrite were selected and analyzed. Various nitrate-reductase-negative mutants led to the identification of a similar nitrate reductase operon, *narGHJI*, as described for *E. coli* (Pantel et al., 1998). Unlike in *E. coli*, in *S. carnosus* there is no evidence for a second operon. The organization of genes and operons involved in dissimilatory nitrate/nitrite reduction are shown in Fig. 8.

Transcription from the *nar* promoter is induced by anaerobiosis, nitrate and nitrite. This is in accordance with the nitrate reductase activities determined with benzyl viologen as electron donor. However, in the presence of oxygen and nitrate, high transcription initiation but low nitrate reductase activity was observed. Since the nitrate reductase formed during anaerobic growth was insensitive to oxygen, other oxygen-sensitive steps (e.g., postranscriptional mechanisms and molybdenum cofactor biosynthesis)

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**A) Genetic organization of the nitrite/nitrate gene locus**

![Genetic organization of the nitrite/nitrate gene locus](image)

- **nirC**, nitrite transporter
- **nir/sir**, nitrite reductase operon
- **nar**, nitrate reductase operon
- **oxygen sensor**, nitrate regulator
- **narT**, nitrate transporter

**B) Genetic organization of the molybdo-cofactor biosynthesis genes**

![Genetic organization of the molybdo-cofactor biosynthesis genes](image)

- **mod**, molybdate transport
- **moa** and **moa** genes most likely involved in biosynthesis of molybdopterin
- **mob** genes most likely involved in insertion of GMP

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Fig. 8. Genetic organization of genes and operons involved in the dissimilatory nitrate/nitrite reduction in *Staphylococcus carnosus*.
must be involved. In *E. coli*, the *nar* promoter is induced by the trans-acting factors ferredoxin-NADP reductase (Fnr) and phosphorylated NarL; Fnr is activated in response to oxygen deprivation and NarL is the response regulator of a two-component system that signals the presence of nitrate. In the *S. carnosus* *nar* promoter, no conspicuous Fnr and integration host factor (IHF) recognition sites are present; there is only one site that is related to the *E. coli* NarL consensus sequence.

**The Nitrite-reducing System**  Characterization of a nitrite-reductase-negative *S. carnosus* Tn917 mutants led to the identification of the *nir* operon, which is comprised of five genes: *nirR*, *sirA*, *nirB*, *nirD* and *sirB* (Neubauer et al., 1999a). The NirBD proteins represent the dissimilatory NADH-dependent nitrite reductase, SirA and SirB (both necessary for biosynthesis of the sioheme prosthetic group), the putative oxidase/chelatase and the uroporphyrinogen III methylase, respectively, and NirR, a protein with known function. It was suggested that NirR is essential for *nir* promoter activity (Neubauer et al., 1999a). In the absence of NirR, a weak promoter upstream of *sirA* seems to drive transcription of *sirA*, *nirB*, *nirD* and *sirB* in the stationary growth phase. Nitrite reduction in *S. carnosus* does not occur in the presence of oxygen and nitrate (Neubauer and Götz, 1996), which is in agreement with the fact that anaerobiosis, nitrite, and nitrate induce the *nir* promoter. Although a transcript is detectable, no nitrite reduction occurs in cells grown aerobically with nitrate or nitrite, indicating an additional oxygen-controlled step at the level of translation, enzyme folding, assembly, or insertion of prosthetic groups. The nitrite-reducing activity expressed during anaerobiosis is switched off reversibly when the oxygen tension increases, most likely owing to the competition for electrons with the aerobic respiratory chain. Another gene, *nirC*, is located upstream of the *nir* operon and encodes a putative integral membrane-spanning protein with unknown function. A *nirC* mutant showed no distinct phenotype. In *S. carnosus*, nitrate is first reduced to nitrite evidently by a dissimilatory enzyme. Nitrite accumulates in the growth medium and is further reduced by a dissimilatory NADH-dependent nitrite reductase. Nitrite reduction only occurs in the absence of nitrate.

The *narT* mutant displayed wildtype levels of nitrate reductase activity but, unlike the wildtype strain, does not take up nitrate and accumulate nitrite when grown in the presence of nitrate under anaerobic conditions (Fast et al., 1997). The 41-kDa NarT is a highly hydrophobic transmembrane protein of 388 amino acids. Its protein sequence shows similarity to *B. subtilis* NasA (25.8% identity) and *E. coli* NarK (22.8% identity).

**Molybdopterin Cofactor Biosynthesis**  All molybdoenzymes except nitrogenase contain a unique form of molybdopterin as cofactor, sometimes conjugated to a nucleoside monophosphate (Rajagopalan and Johnson, 1992). At least five different loci in *E. coli* are involved in molybdate transport and processing, synthesis of molybdopterin, and conversion to the dinucleotide form (Rajagopalan, 1996).

In *S. carnosus*, nine genes were identified (Fig. 8), all of which appear to be involved in molybdenum cofactor biosynthesis (Neubauer et al., 1998). The regulation and function of one of the proteins, MoeB, was further analyzed. In addition, *moeB* Tn917-insertion mutants showed no nitrate reductase activity because they were molybdenum-cofactor-deficient. However, nitrate reductase activity in cell-free extracts could be reconstituted with a low-molecular-weight component (most likely free molybdenum cofactor) from an *S. carnosus* mutant that is defective in the nitrate reductase structural genes. Expression studies of *moeB* indicated that anaerobiosis and nitrate each enhance transcription of *moeB* (Neubauer et al., 1998).

**Molybdate Transport System**  *ModABC*  In *S. carnosus* three genes, *modABC*, were identified which encode an ATP-binding cassette (ABC) transporter that is involved in molybdate transport (Neubauer et al., 1999b). These genes are located directly upstream of *moeB* and seem to be part of the molybdenum cofactor biosynthesis gene cluster. The *mod* mutants are devoid of nitrate reductase activity. It was shown by 14C-palmitate labeling that ModA represents a lipoprotein that, in Gram-positive bacteria, is the counterpart of the periplasmic binding proteins of Gram-negative organisms. The sequence characteristics identify ModB as the integral-membrane, channel-forming protein and ModC as the ATP-binding energizer for the transport system. Mutants defective in *modABC* revealed only 0.4% of wildtype nitrate reductase activity. Molybdate at a nonphysiologically high concentration (100 μM) fully restored nitrate reductase activity, suggesting that at least one other system is able to transport molybdate but with lower affinity. The expression of *modA* (and most likely *modBC*) was independent from oxygen and nitrate. To date, there are no indications for molybdate-specific regulation of *modABC* expression since in a *modB* mutant, *modA*
expression was unchanged and no different from that in the wildtype strain (Neubauer et al., 1999b).

Recently in \textit{S. carnosus}, the \textit{nreABC} (for nitrogen regulation) genes were identified and shown to link the nitrate reductase operon (\textit{nargHJI}) and the putative nitrate transporter gene \textit{nart}. An \textit{nreABC} deletion mutant was dramatically affected in nitrate and nitrite reduction and growth. The data provide evidence for a global regulatory system important for aerobic and anaerobic metabolism, with NreB and NreC forming a classical two-component system and NreB acting as a sensor protein with oxygen as the effector molecule (Fedtke et al., 2002).

Homologous sequences of the described nitrate/nitrite reductase systems in \textit{S. carnosus} are also identifiable in the \textit{S. aureus} N315 and \textit{S. epidermidis} RP62A genomes. Thus the ability to use nitrate as an alternative terminal electron donor appears to be widely distributed among staphylococci.

Cell Wall Peptidoglycan

\textit{Staphylococcus aureus} was one of the first bacterial species where peptidoglycan biosynthesis was studied and pioneering results achieved. A comprehensive description is presented in the monographs by Rogers et al. (1980b) and Ghuysen and Hakenbeck (1994). The staphylococcal peptidoglycan is a heteropolymer consisting of glycan strands crosslinked by peptides. According to the classification by Schleifer and Kandler (1972), \textit{S. aureus} has an \textit{A3\alpha} peptidoglycan type. The peptide chain length is generally long in Gram-positive bacteria, yet the most extensively crosslinked peptidoglycan, that of \textit{S. aureus}, has an average of about 15 repeating units and a maximal chain length of 30–40 peptide units (Snowden and Perkins, 1990; Henze et al., 1993). In exponentially grown cells in rich medium, more than 95% of the subunits are crosslinked in \textit{S. aureus}. This extremely high degree of crosslinking is possible only because their long and flexible pentaglycine interpeptide bridges are able to span distances between peptides otherwise much too far apart to be crosslinked (Ghuysen and Hakenbeck, 1994).

The peptidoglycan of staphylococci is also exceptional in that, apart from the small proportion of residual uncross-linked primary pentapeptide side-chains, it has almost no free carboxyl groups, since the D-glutamic acid \(\alpha\)-carboxyl group is amidated (Tipper et al., 1967; Schleifer, 1975a; Pucci et al., 1995).

\textit{Staphylococcus aureus} also provides an example of the second type of peptidoglycan modification, the presence of \textit{O}-acetyl substituents on the \textit{N}-acetylmuramidic acid residues. In this organism about 50% of the muramic acid residues are present as the \textit{4-N, 6-O-diacetylderivative} (Tipper et al., 1971). This substitution has the effect of making the peptidoglycan resistant to the muramidase egg-white lysozyme (Warren and Gray, 1965).

Additions to the peptidoglycan of \textit{S. aureus} also occur in the form of covalently linked wall teichoic acid. They are attached by phosphodiester linkages on \(C_\delta\) of some of the muramic acid residues (Hay et al., 1965). In \textit{S. aureus}, approximately 7.7% of the muramic acid is phosphorylated. Suggesting that on approximately every thirteenth muramic acid a wall teichoic acid is covalently linked.

As in all staphylococci, consecutive cell divisions are initiated at an angle of 90 degrees in three dimensions, sometimes even before completion of the cell separation process of the first division plane. Cell division is achieved by the formation of a highly organized cross wall, which is initiated asymmetrically and eventually fuses in the center of the cell to form a complete cross wall. On the basis of distinct morphological features, as revealed by electron microscopy, involvement of several autolytic systems has been suggested in cell division process. One of these, the splitting system, appears as a ring of periodically arranged tubules in the center of the cross wall (Giesbrecht et al., 1998).

Teichoic acid-like material has been chemically associated with the splitting system (Morioka et al., 1987). During isolation and purification of the peptidoglycan, the morphological appearance of the splitting system remains detectable until the final purification step, i.e., removal of the teichoic acid. If the splitting system can no longer be detected, two separated pieces of cross wall are visible. Once the cross wall has been completed, cell separation is initiated by highly organized entities called “murosomes,” which punch tiny holes into the peripheral wall along the division plane (Giesbrecht et al., 1998).

**BIOSYNTHESIS OF PEPTIDOGLYCAN**

The overall peptidoglycan synthesis can be divided into three distinct stages (Rogers et al., 1980b): 1) The formation of the nucleotide sugar-linked precursors, UDP-\textit{N}-acetylmuramic, UDP-\textit{N}-acetylmuramic acid and UDP-\textit{N}-acetylmuramyl-pentapeptide; 2) the transfer of phospho-\textit{N}-acetylmuramyl-pentapeptide and \textit{N}-acetylmuramic acid to the lipophilic carrier, undecaprenyl phosphate, to yield a disaccharide-(pentapeptide)-pyrophosphate-undecaprenol; and 3) the transfer of this complete subunit to the growing peptidoglycan. At this stage, cross-bridge formation occurs together with secondary modification of the newly synthesized peptidoglycan. Known genes
(enzymes) involved in the pathway of peptidoglycan biosynthesis are shown in Fig. 9, and in Table 12, annotated peptidoglycan biosynthesis genes of S. aureus N315 are listed.

UDP-N-acetylmuramic acid is formed from UDP-N-acetylgalactosamine (UDP-GlcNac), UTP and N-acetylglucosamine-1-P. The synthesis of UDP-GlcNac starts from fructose-6-P and involves UTP-glucose-1-P uridylyltransferase (GtaB), glucosamine-fructose-6-P aminotransferase (GlmS), and phosphoglucosamine-mutase GlmM (FemD). The UDP-N-acetylgalactosaminepyrophosphorylase reaction is also involved in the synthesis of many other nucleotide-linked sugars. The next step is the formation of the first intermediate UDP-N-acetylmuramic acid. These reactions involve the transfer of a pyruvate enol ether from phosphoenolpyruvate to UDP-N-acetylgalactosamine and its subsequent reduction to yield UDP-N-acetylmuramic acid (Wickus et al., 1973). The enzyme involved is UDP-N-acetylmuramic acid 1-carboxyvinyltransferase. In the genome sequence, there are two transferases present, MurA and MurZ (MurB). The transferase activity was initially detected in cell-free

Table 12. Annotated peptidoglycan biosynthesis genes of S. aureus N315.

| Gene designation | Encoded enzyme |
|------------------|----------------|
| glmS             | Glucosamine-fructose-6-phosphate aminotransferase |
| glmM (femD)      | Phosphoglucomamine-mutase |
| gtaB             | UTP-glucose-1-phosphate uridylyltransferase |
| murA             | UDP-N-acetylgalactosamine 1-carboxyvinyl transferase 1 |
| murZ             | UDP-N-acetylgalactosamine 1-carboxyvinyl transferase 2 |
| murC             | UDP-N-acetylmuramic acid-alanine ligase |
| ddlA             | D-Alanine-D-alanine ligase |
| murF             | UDP-N-acetylmuramoylalanine-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanyl ligase |
| murI             | Glutamate racemase |
| murD             | UDP-N-acetylmuramoylalanine-D-glutamate ligase |
| mraY             | Phospho-N-muramic acid-pentapeptide translocase |
| mraG             | Undecaprenyl-PP-MurNAc-pentapeptide-UDPGLcNAc GlcNAc transferase |
| murE             | UDP-N-acetylmuramoylalanine-D-glutamate-2,6-diaminopimelate ligase |
| sgtA             | Probable transglycosylase |
| sgtB             | Hypothetical protein, similar to penicillin-binding protein 1A/1B |
| uppS             | Undecaprenyl pyrophosphatase synthetase |
| php4             | Penicillin binding protein 4 |
| bacA             | Bacitracin resistance protein (putative undecaprenol kinase) homologue |
| femA             | Factor essential for expression of mexiticin resistance |
| femB             | FemB protein |
| uppS             | Undecaprenyl pyrophosphatase synthetase |
| phpA             | Penicillin-binding protein 1 |
| php2             | Penicillin-binding protein 2 |
| php3             | Penicillin-binding protein 3 |
| sgtB             | Hypothetical protein, similar to penicillin-binding protein 1A/1B |
| mraY             | Similar to UDP-N-acetylgalactosamine pyrophosphorylase |
| fnhA             | FmhA protein |
| fnhB             | FmhB protein |
| fnhC (eprh)      | FmhC protein |
| srtA             | Sortase |
| drp35            | Drp35 |
| ebsB             | Cell wall enzyme |

From Kuroda et al. (2001).
extracts from *S. aureus* (Strominger, 1965). It was partially purified from *S. epidermidis* (Wickus et al., 1973). The reaction is reversible and moreover specifically and irreversibly inhibited by fosfomycin. The UDP-GlcNAc-enolpyruvate reductase was also purified from *S. aureus* (Wickus et al., 1973). In all cases, NADPH is used as co-factor. The synthetases that catalyze the assembly of the peptide moiety of the peptidoglycan unit are cytoplasmic.

The UDP-MurNAc pentapeptide is formed by the sequential addition of L-alanine, D-glutamic acid, L-lysine and D-alanyl-D-alanine to the D-lactyl group of UDP-MurNAc. Each step is catalyzed by a specific synthetase using ATP. The involved enzymes are MurC, MurD, MurE and DdlA (see Table 9). The end product is UDP-MurNAC-L-Ala-D-iso-Glu-L-Lys-D-Ala-D-Ala (also named “UDP-MurNAc-pentapeptide” and “Park’s nucleotide”).

The conversion of L-Glu to D-Glu is catalyzed by MurI. The conversion of L-Ala to D-Ala is catalyzed by alanine racemase, an enzyme studied in detail in *S. aureus* (Cheung et al., 1983; Kullik et al., 1998b). D-Alanyl-D-alanine is formed by the dimerization of D-alanine. The enzyme involved, D-alanine:D-alanine ligase, has been already purified from *S. aureus* by Ito and Strominger (1973). The enzyme requires a “heat-stable co-factor,” Mg²⁺ (or Mn²⁺), K⁺ and ATP for activity (for a review, see Rogers et al., 1980a).

Under certain conditions, glycine has been shown to replace both alanine isomers in UDP-N-acetyl muramyl pentapeptide. Growth of *S. aureus* in the presence of high concentrations of glycine resulted in the accumulation of modified UDP-N-acetylmuramyl-pentapeptides containing both glycyl-D-alanine and D-alanyl-glycine as the terminal dipeptide (Hammes et al., 1973). Thus, under these rather specific conditions the substrate specificities of both the D-alanine ligase and the UDP-N-acetylmuramyl-3-peptide:D-Ala-D-Ala-ligase have been overridden. The synthesis and subsequent incorporation into peptidoglycan of such natural precursors may contribute to the inhibitory effects of glycine and D-amino acids on many bacteria.

**Biosynthesis of the Lipid II Precursors**

Undecaprenol, originally present either as the free alcohol or as a phosphorylated derivative has been isolated in pure form from *S. aureus* (Higashii et al., 1970). The 17-kDa phosphokinase of *S. aureus* is soluble in several organic solvents but insoluble in water and has an unusually high content (58%) of nonpolar amino acids. Subsequent purification allows the separation of an apoprotein to which activity can be restored by a wide range of natural and synthetic phospholipids and detergents. Initially it was suggested that phosphatidyglycerol or diphasphatidyglycerol (cardiolipin) were specific lipid co-factors. However, enzymatic activity appears to be more dependent on “lipid hydration” rather than lipid viscosity or the actual chemical structure of the polar group of the activating lipid (Sanderman, 1976). In stationary-phase cultures, free undecaprenol was present in large excess, representing approximately 80% of the total peptidoglycan lipid.

UDP-MurNAc-pentapeptide is phosphodiesters linked to an undecaprenyl-pyrophosphate carrier molecule at the expense of UDP to yield C₅₅–PP-MurNAC-L-Ala-D-Gln-L-Lys-D-Ala-D-Ala, or lipid I (Higashii et al., 1970). UDP-GlcNAC is linked to the muramoyl moiety to generate the disaccharide lipid II precursor [C₅₅–PP-MurNAC(-L-Ala–D-Gln–L-Lys(Gly5)-D-Ala-D-Ala)]–β1-4-GlcNAc]. Lipid I and lipid II precursors are hooked to the inner side of the cytoplasmic membrane where also very likely the synthesis of the pentaglycine interpeptide takes place. Recently it was shown that lipid II is a peptidoglycan substrate for sortase-catalyzed surface protein anchoring (Perry et al., 2002).

**Formation of Pentaglycine Interpeptide Synthesis**

of the pentaglycine chain occurs at the membrane-bound lipid II precursor GlcNAC(-β1,4)-N-acetylmuramic acid-(L-Ala–D-Gln–L-Lys–D-Ala–D-Ala)-pyrophosphoryl-undecaprenol by sequential addition of glycine to the ε-amino group of lysine, using glycyI-tRNA as donor, in a ribosome independent fashion (Kamiyoy and Matsuhashi, 1972). First hints for an involvement of glycyI-tRNA were based on the finding that incorporation of glycine into polymeric peptidoglycan was prevented by ribonuclease. Under identical conditions, the incorporation of N-acetylmuramyl-peptide from the nucleotide precursor was unaffected. Five glycine residues activated by tRNA are added initially to the ε-NH₂ group of lysine and then subsequently to the N-terminus of the growing peptide chain (Kamiyoy and Matsuhashi, 1972). This mechanism is in direct contrast to that of protein synthesis, which occurs by addition at the carboxyl terminus. The residues appeared to be added singly with no evidence for the involvement of peptide tRNA intermediates. The tRNA of *S. aureus* has been found to contain at least three species of glycyI-tRNA (Gly-I-tRNA). They can be used for the incorporation of glycine into peptidoglycan, whereas one of the species is inactive in protein synthesis. The formation of all Gly-tRNA is catalyzed by a single Gly-tRNA synthetase, which has been purified (Niyomporn et al., 1968). A more complex situation has been found
in *S. epidermidis* where four different pentapeptide cross-bridges have been shown to be present (Tipper and Berman, 1969). Each contains glycine and L-serine residues in the ratio 3:2 in a characteristic sequence where glycine is always the initial substituent of the ε-amino group of L-lysine. Addition of the amino acids occur at the level of lipid intermediates, the transfer of glycine and serine from their respective tRNAs being catalyzed by membrane-bound enzymes. The incorporation of glycine is independent of that of serine, whereas maximum serine incorporation requires the simultaneous incorporation of glycine. Fractionation of tRNA of *S. epidermidis* has demonstrated the presence of four glycyland four seryl-tRNAs. In each case, all species, including one species of each type that did not participate in protein synthesis, were active in cross-bridge synthesis. The glycyyl-tRNA with apparent specificity for peptidoglycan synthesis has been purified and shown to be made up of two distinct iso-accepting species (Roberts et al., 1974). The two sequences define six spaces and the insertion of an additional base in the dehydrouridine loop of one. Thus, *S. epidermidis* appears to have developed a means of supplying glycine residues for cross-bridge synthesis without involving the machinery of protein synthesis.

**The Fem Factors** The expression of methicillin resistance depends on several genes named “*fem*” (factors essential for methicillin resistance; Berger-Bächi, 1994). Recently, it was demonstrated that FmhB (FemX) links the first glycine residue to the ε-amino group of lysine (Rohrer et al., 1999). Evidence for this function was that, under conditions where *fmhB* is downregulated, unhydrolysed peptidoglycan monomers accumulate. Since FmhB is essential in *S. aureus*, it represents an ideal drug target. While FmhB incorporates the first glycyyl residue, FemA incorporates glycyyl residues 2 and 3 (Stranden et al., 1997), and FemB, glycyyl residues 4 and 5 (Henze et al., 1993). Although FemA and FemB have a relatively high amino acid sequence identity (40%) and similarity (64%), they are specific and cannot substitute for each other. Null mutants of *femAB* are barely viable and depend on compensatory mutations for survival (Ling and Berger-Bächi, 1998). Interestingly, *S. aureus* contains one glycyyl-tRNA gene for protein biosynthesis and three non-proteinogenic glycyyl-tRNA genes that are involved in cell wall biosynthesis (Green and Vold, 1993). It is tempting to speculate that the two Fem factors and FmhB may preferentially recognize one of the three glycyyl-tRNA species. The site of glycine incorporation in the peptidoglycan is shown in Fig. 10.

Other FemAB-like factors have been identified in staphylococci, such as lysostaphin immunity factor (Lif) in *S. simulans* var. *staphyloccicus* and is responsible for incorporation of serine residues in positions 3 and 5. Lif, lysostaphin immunity factor; MurNAc, N-acetylmuramic acid; and GlcNAc, N-acetylglycosamine.

The expression of *lif* in *S. carnosus* led to an increase of the serine-glycine ratio of the interpeptide bridges from 2 to 35%, suggesting that lysostaphin immunity depends on serine incorporation into the interpeptide bridge. If in addition to *lif*, *lss* is coexpressed, the serine-glycine ratio is further increased to 58%, suggesting that Lss selects for optimal serine incorporation. Lif shows similarity to FemA and FemB proteins, which are involved in the biosynthesis of the glycine interpeptide bridge of staphylococcal peptidoglycan. In contrast to Lif, the production of FemA and FemB in *S. carnosus* does not cause lysostaphin immunity (Thumm and Götz, 1997). The putative tRNA<sup>Ser</sup> gene located downstream of *lss* had no recognizable influence on lysostaphin immunity. The *lss* and *lif* genes are flanked by insertion sequences, suggesting that *S. simulans* biobar *staphylococcus* received *lif* and *lss* by horizontal gene transfer. Recently, it was shown that Lif and Epr lead to incorporation of serine residues into the staphylococcal peptidoglycan interpeptide bridges specifically at positions 3 and 5 and that this incorporation requires the presence of FemA and/or FemB (Ehler et al., 2000).

**Translocation of Lipid II-Pentaglycine** In the second stage of the biosynthetic process, the lipid II-pentaglycine is translocated from the inner side of the cytoplasmic membrane to the outer side where the incorporation into the growing peptidoglycan occurs. The initial reaction...
Involves the transfer of phospho-N-acetylmuramyl-pentapeptide from the nucleotide to undecaprenyl phosphate with the formation of undecaprenyl-P-P-N-acetylmuramyl-pentapeptide and UMP. This reaction is catalyzed by a translocase. Incorporation of undecaprenylpyrophosphate in UDP-N-acetylmuramyl-pentapeptide is inhibited by tunicamycin. In a second step N-acetyl-glucosamine is glycosidically linked to the N-acetyl-muramyl residue to form undecaprenyl-P-P-N-acetylmuramyl (pentapeptide)-N-acetylglucosamine and UDP (Anderson et al., 1967). Since these reactions involve both hydrophilic and hydrophobic substrates, it seems likely that both enzymes act at the cytoplasmic lumen of the membrane.

The translocase (phospho-N-acetylmuramyl-pentapeptide translocase), which has been extensively studied by Neuhaus and his colleagues (Pless and Neuhaus, 1973), can be conveniently assayed either in the forward direction as a transferase or in the reverse direction, by the exchange reaction following the incorporation of radioactivity from UMP into UDP-N-acetylmuramyl-pentapeptide. The enzyme has been obtained in soluble form from S. aureus by treatment of membrane preparations with the non-ionic detergent Triton X-100 (Pless and Neuhaus, 1973; Weppner and Neuhaus, 1977). The enzyme is stimulated by the addition of polar lipid fraction containing phosphatidylglycerol. However, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol were equally capable of restoring enzyme activity. Thus, the reactivation of the translocase appeared to result from the provision of a lipid microenvironment rather than a requirement for a specific phospholipid. In vivo, the microenvironment of the translocase is the lipids of the membrane with which it interacts.

Investigation of the microenvironment in membranes of the fluorescent lipid intermediate undecaprenyl-N-acetylmuramyl-(N-dansyl)-pentapeptide suggests that the lipid intermediate is immobilized within hydrophobic environment close to the membrane surface. This observation is supported by the earlier finding that spin-labelled lipid intermediate could still form complexes with vancomycin and ristocetin (Johnston and Neuhaus, 1975). It was proposed that at least the terminal D-alanyl-D-alanine-dipeptide of the pentapeptide side chain has access to the aqueous phase. Vancomycin and ristocetin mimics the effect of detergents. At low concentrations, the antibiotic stimulates the transfer but not the exchange reaction. Hammes and coworkers (Hammes and Neuhaus, 1974) have investigated the specificity of phospho-N-acetylmuramyl-pentapeptide translocase from S. aureus. They could show that the translocase has 24% activity when the terminal D-alanine is missing, 60% activity when the terminal amino acid is glycine instead of D-alanine, and 57% activity when meso-diaminopimelic acid (mDAP) instead of L-lysine is present.

In many organisms, the lipid-linked disaccharide pentapeptide units are utilized directly for synthesis of peptidoglycan. In S. aureus, the carboxyl group of D-isoglutamic acid is amidated. The reaction is catalyzed by a membrane-bound enzyme. The enzyme can utilize either glutamine or NH₄⁺, and both undecaprenyl-P-P-N-acetylmuramyl-pentapeptide and disaccharide-pentapeptide act as substrates, whereas a nucleotide precursor UDP-N-acetylmuramyl-pentapeptide is completely inactive. The reaction involves hydrolysis of ATP to ADP. It seems likely that many other minor differences found in the structure of peptidoglycan such as the O-acetylation of muramic acid residues in S. aureus (Ghuyse and Hakenbeck, 1994) and the addition to the peptide side chain of single amino acid residues, not involved in interpeptide-bridge formation, occurs at the level of lipid intermediates.

The final reaction of the membrane-mediated stage of peptidoglycan synthesis is also the initial reaction in the third stage, i.e., the incorporation of the newly synthesized disaccharide peptide unit into the growing peptidoglycan. The presence of uncross-linked side chains in all peptidoglycans examined argues strongly that polymerization occurs by extension of the glycan chain (transglycosylation) prior to peptide-bond formation (transpeptidation). The finding of free reducing groups of muramic acid in the walls of S. aureus (Ward and Perkins, 1973) suggests that there must exist some mechanisms to terminate synthesis of a particular glycan chain by detaching the lipid carrier. At present, the nature of this process is not evident.

**Transpeptidation: The Formation of Crosslinks** By the mid-1960s, detailed studies of the chemical structure of several peptidoglycans established the presence of crosslinked peptides. It was suggested that D-alanyl-D-alanine-dipeptide appeared to be involved in the formation of these cross-linkages. When sublethal concentrations of penicillin were applied, the walls contained an increase in the free amino groups of glycine and an excess of alanine over those walls prepared from organisms grown in the absence of penicillin. These observations were independently confirmed by direct chemical analysis of staphylococcal walls prepared from organisms grown under similar conditions (Tipper and Strominger, 1968). In the presence of penicillin, an increased synthesis of uncross-
linked peptide monomer units was observed. These units retained the complete D-alanyl-D-alanine terminus of the nucleotide precursor and were substituted with the pentaglycine cross-bridge peptide on the ε-amino group of L-lysine. During subsequent growth of the organism in the absence of penicillin, these monomer units did not become crosslinked. To explain these observations, it was proposed that incorporation of the newly synthesized disaccharide-peptide occurred at the wall-membrane interface with almost immediate formation of crosslinks to adjacent peptide side chains. In staphylococci, units synthesized in the presence of penicillin could not form crosslinks on the subsequent removal of the antibiotic because continued synthesis of linear uncross-linked peptidoglycan during the initial penicillin treatment moved these units outside the reach of the transpeptidation molecules. These in vivo studies supported the conclusion that crosslinking in peptidoglycan occurred by means of a transpeptidation reaction inhibited by β-lactam antibiotics.

In vitro, cross-link studies with cell wall preparations from *S. aureus* (Mirelman and Sharon, 1972) were shown to retain some cytoplasmic membrane not removed by extensive washing. These preparations catalyzed the incorporation of radioactivity from UDP-N-acetylMuramyl[14C]-L-lysine)pentapeptide into both peptidoglycan and lipid intermediates. The penicillin sensitive release of D-alanine occurred when the wall-membrane preparation was incubated with the two nucleotide precursors and glycine, whereas no such release occurred in the preparations incubated in the absence of UDP-N-acetylglucosamine. Glycine incorporation was sensitive to ribonuclease. Penicillin also inhibited the incorporation of glycine, but in contrast to release of D-alanine, this was never complete.

**Autolysins and Turnover of Bacterial Wall Polymers** There are various autolytic enzymes present in most staphylococci:

1) An enzyme that hydrolyzes the N-acetylMuramyl-1,4-β-N-acetylglucosamine bonds in the glycans to liberate free-reducing groups of N-acetylmuramic acid; since this enzyme is only active with microccocal but not with staphylococcal peptidoglycan, we postulate that it plays a role before the peptidoglycan becomes O-acetylated.

2) A β-N-acetylglucosaminidase that liberates the free reducing groups of N-acetylglucosamine

3) An N-acetylmuramyl-L-alanine amidase (amidase) that hydrolyzes the bond between the glycan chains and the peptide subunits

4) Endopeptidases that can hydrolyze some of the main peptides and the bridge peptide when they occur between the C-terminal D-alanine and the amino group of a contiguous peptide chain

5) Commonly occurring in many organisms are D-alanine-carboxypeptidases that can cleave off any carboxyl terminal D-alanyl residues of the peptidoglycan. These enzymes, however, are not known to act as autolysins and therefore will not be considered.

Staphylococcus aureus does not exhibit a lysozyme like muramidase (EC 3.2.1.17), which cleaves its O-acetylated peptidoglycan.

Pulse chase studies indicated that walls of many organisms such as bacilli, lactobacilli or staphylococci are in a state of flux. Wall synthesis and degradation are balanced by a so-called “turnover of the cell wall.” If wall synthesis is inhibited in such situations, without a corresponding inhibition of either the formation or action of the autolysins, cell lysis might be expected and indeed occurs. Antibiotics that inhibit wall synthesis are bactericidal, unlike most of those inhibiting protein synthesis, which are bacteriostatic.

There are still several open questions: Is the inhibition of wall synthesis sufficient to kill bacteria? Do cells lyse because their walls can no longer expand so that the cell bursts? Is autolytic action essential? One possible way to answer these questions would be to stop the formation of autolysins by blocking protein synthesis. A combination of chloramphenicol and penicillins is not bactericidal for *E. coli* or for staphylococci (Rogers et al., 1980b). Lysis of staphylococci could also be stopped, as with *B. subtilis* and *Enterococcus faecalis*, by the inhibition of protein synthesis. However, to date the answers to these questions have been inconclusive. The observation that an autolytic deficient strain of *S. aureus* failed to show wall turnover, although the wildtype turnover rate was rather slow (Chatterjee et al., 1976) should be reevaluated.

Suginaka et al. (1979) have shown that the addition of homologous lipoteichoic acid to cultures of *S. aureus* treated with penicillin prevents lysis and death of the bacteria. Forsman antigen (lipoteichoic acid from pneumococci) can also prevent penicillin-induced lysis in *S. aureus.* Lipoteichoic acids or lipids certainly regulate the action of autolysins in vivo. It was also found that wall autolysis is inhibited by sodium polyanethole sulfonate “liquoid” (Wecke et al., 1986). The inhibition is not due to direct interaction with the autolysins, and liquid blocks very likely the target structures of autolysins, especially the wall teichoic acid (WTA).
Biological Activity of Peptidoglycan  It was early recognized that cell wall muramyl peptides exert excellent adjuvant properties, and consequently a variety of muramyl derivatives with biological potentials as adjuvant have been synthesized and investigated (Adam et al., 1975). In a somewhat analogous manner, another interesting biological property (i.e., the somnogenic effects of muramyl compounds) was investigated by Krueger and coworkers (Johannsen et al., 1989; Johannsen et al., 1991a; Johannsen et al., 1991b). It is of interest that macrophages produce somnogenic and pyrogenic muramyl peptides during digestion of staphylococci. Somnogenic compounds of bacterial origin are obviously small enough to cross the blood-brain barrier, and it is of interest to speculate that lysozyme capable of digesting the insoluble wall peptidoglycan polymer could have a role in the production of suitably sized substances with such properties.

Obviously the immune system recognizes that muramyl peptides are products of bacteria, and responds by becoming activated to resist infection. This resistance to infection is nonspecific and extends to unrelated species of bacteria, fungi, and viruses. A key mechanism of the resistance to infection is activation of macrophages. Macrophage activation results in increased production of microbicidal oxygen radicals like superoxide and peroxide, and in increased secretion of inflammatory cytokines like interleukin-1β and tumor necrosis factor-α. These cytokines, besides activating neutrophils, B lymphocytes, and T lymphocytes, act on the central nervous system to induce physiological responses like fever and sleep. These physiological responses also aid in combating infection. Muramyl peptides activate macrophages and other cells of the immune system to kill cancer cells. It is therefore believed that muramyl peptides and similar agents will become more important as therapeutics in the future, owing to increasing resistance of microbes to antibiotics, and increasing numbers of patients with immunodeficiencies (Pabst et al., 1999).

Mammals have a peptidoglycan recognition protein (PGRP) which binds to peptidoglycan (PG) or live bacteria and which is upregulated by PG. This PGRP is ubiquitous and involved in innate immunity. Tag7, a novel cytokine, is also induced by bacterial products; Tag7 is apoptotic to murine L929 cells in a NF-kB-independent manner. Both the PGRP and tag7 genes are expressed in brain, lymphatic and hematopoietic tissues. Recently, it was found that murine PGRP and tag7 encode identical transcripts, and their corresponding proteins have structural relationships to lysozymes. Furthermore, the cDNA of rat PGRP was cloned and expressed in brains of sleep-deprived and control rats. It turned out that PGRP expression was upregulated by sleep deprivation, suggesting a role for PGRP in a homeostatic regulation of sleep (Rehman et al., 2001).

Antibiotics Affecting Bacterial Peptidoglycan Synthesis

Phosphonomycin (Fosfomycin)  This antibiotic, the isolation and characterization of which were first described in 1969, inhibits what might be regarded as the earliest step in peptidoglycan synthesis. It inhibits the transfer of the enolpyruvate residue from phosphoenol pyruvate to UDP-N-acetylglucosamine (UDP-N-acetylgulosamine-3-O-enolpyruvyl transferase). Phosphonomycin binds covalently to cysteine residues of the transferase. Resistance is achieved by mutations in the transport systems. For review of the cell wall antibiotics, see Rogers et al. (1980a).

Cycloserine, O-Carbamoyl-D-serine, Alaphosphin, and the Haloalanines  Antibiotics that inhibit D-alanine metabolism are cycloserine, O-carbamoyl-D-serine, alaphosphin (L-alanyl-L-1-aminoethyl phosphonic acid) and the haloalanines. Alanine racemase is competitively and irreversibly inhibited by cycloserine, O-carbamoyl-D-serine and the haloalanines.

The inhibiting effect of D-cycloserine on bacterial growth can be reversed by the addition of D-alanine. The presence of D-alanine prevents conversion to spheroplasts of several bacteria incubated with cycloserine in osmotically stabilized media. Cycloserine acts by inhibiting the alanine racemase and thus the incorporation of D-alanine into the peptidoglycan. As a consequence, UDP-N-acetylumarmyl-L-alanine-D-isoglutamyl-L-lysine accumulates. O-carbamoyl-D-serine, alaphosphin (L-alanyl-L-1-aminoethyl phosphonic acid) and the haloalanines act in a similar way. While D-cycloserine alone appears to inhibit D-alanine:D-alanine ligase, the D-alanyl-D-alanine adding enzyme (UDP-N-acetylumarmyl-L-alanine-D-isoglu-L-lys:D-Ala-D-Ala ligase [ADP]) is not affected by any of these antibiotics. D-Cycloserine is a competitor of the substrate for both alanine racemase and D-alanine:D-alanine ligase. The inhibition constant of D-cycloserine for alanine racemase is $5 \times 10^{-5}$. Resistance to D-cycloserine can arise either by elevated racemase and ligase activity or by inability to transport the antibiotic.

Bacitracin  Bacitracin specifically inhibits the dephosphorylation of undecaprenyl-pyrophosphate.
**Vancomycin and Ristocetin B** Inhibition occurs very rapidly and binding of the antibiotic to bacteria takes place within 20 seconds of treatment. In *S. aureus*, approximately $10^7$ molecules are bound per cell (although this number might be too high because of aggregate formation). Both vancomycin and ristocetin B bind preferentially to the terminal D-alanyl-D-alanine peptide of the UDP-N-acetylglucosamine-pentapeptide. The binding site of ramoplanin appears to be different from that of vancomycin (Nieto et al., 1972; Somner and Reynolds, 1990).

Glycopeptide resistance in *S. aureus* is poorly understood. Characterization of vancomycin-resistant *S. aureus* (VRSA) strains from all over the world confirmed that emergence of vancomycin resistance in *S. aureus* is a global issue. A certain group of *S. aureus*, designated “hetero-VRSA,” frequently generates VRSA upon exposure to vancomycin, and is associated with infections that are potentially refractory to vancomycin therapy (Hiramatsu, 2001). Vancomycin resistance is acquired via mutation and cell wall thickening, due to accumulation of excess amounts of peptidoglycan. This seems to be a common resistance mechanism for all VRSA strains isolated so far (Geisel et al., 2001; Komatsu et al., 2002). By exposure to vancomycin, a resistant *S. aureus* COL mutant was isolated. This mutant showed decreased susceptibility to teicoplanin (8-fold), methicillin (2-fold), macarboymycin (8-fold), and moenomycin (16-fold). Macarboymycin and moenomycin are thought to directly inhibit transglycosylase activity. Characterization of the mutant revealed a thickened cell wall and suppression of penicillin-induced lysis, although the amounts of the five penicillin-binding proteins (PBPs 1, 2, 3 and 4) and MecA, and the profiles of peptidoglycan hydrolases were not altered. Analysis of muropeptide profile and glycan chain length distribution by reverse-phase high-pressure liquid chromatography revealed slightly decreased peptide crosslinking and an increased average glycan chain length compared to those of the parent. These results together suggest that a transglycosylase activity was enhanced in the mutant. This may represent a novel mechanism of glycopeptide resistance in *S. aureus* (Komatsu et al., 2002).

In June 2002, a VRSA was isolated from a swab obtained from a catheter exit site of a Michigan resident aged 40 years with diabetes, peripheral vascular disease, and chronic renal failure. The patient received dialysis at an outpatient facility (Centers for Disease Control and Prevention, 2002). In June, the patient developed a suspected catheter exit-site infection, and the temporary dialysis catheter was removed; cultures of the exit site and catheter tip subsequently grew *S. aureus* resistant to oxacillin (minimal inhibitory concentration [MIC] > 16 µg/ml) and vancomycin (MIC > 128 µg/ml). It turned out that the isolate contained the vanA vancomycin resistance genes from enterococci, which is consistent with the glycopeptide MIC profiles. Transfer of vanA genes from *Enterococcus faecalis*, with which the patient was also infected, to *S. aureus* is strongly indicated. And to make it worse, the *S. aureus* isolate contained in addition the oxacillin/methicillin-resistance gene mecA. This report describes the first clinical isolate of *S. aureus* that is fully resistant to vancomycin (Centers for Disease Control and Prevention, 2002). Transfer of van resistance genes from enterococci to *S. aureus* has long been predicted, with such a conjugative transfer having been demonstrated in vitro (Noble et al., 1992).

**β-lactam and Methicillin Resistance** Three basic enzymes have been described which are subject to penicillin-inhibition: transpeptidases, D-alanine carboxypeptidases, and endopeptidases. The blanket resistance of methicillin-resistant *S. aureus* to all β-lactam antibiotics is related to the properties of the key component of this resistance mechanism: the “acquired” penicillin-binding protein MecA, which has unusual low affinity for all β-lactam antibiotics. Until now, the accepted model of resistance had implied that in the presence of β-lactam antibiotics, MecA must take over the biosynthesis of staphylococcal cell wall from the four native staphylococcal PBPs (PBP1, PBP2, PBP3 and PBP4) because the latter become rapidly acylated and inactivated at even low concentrations of the antibiotic. However, recent observations indicate that this model requires revision. Inactivation of the transglycosylase domain, but not the transpeptidase domain, of MecA of *S. aureus* prevents expression of β-lactam resistance, despite the presence of the low-affinity MecA. The observations suggest that cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the transglycosylase domain of the native staphylococcal PBP2.
and the transpeptidase domain of the MecA (Pinho et al., 2001). There are indications that the meca gene was acquired from S. sciuri which is, however, uniformly susceptible to β-lactam antibiotics. Recently, it was shown that the meca homologue in S. sciuri is hardly expressed but is well expressed by a single mutation in the promoter region and provides methicillin resistance in a susceptible S. aureus strain. This supports the proposition that the meca homologue ubiquitous in the antibiotic-susceptible animal species (S. sciuri) may indeed be an evolutionary precursor of the methicillin resistance gene meca of the pathogenic strains of MRSA (Wu et al., 2001).

The peptidoglycan isolated from methicillin resistant S. aureus (MRSA) strains that contain meca did not differ from that of the susceptible S. aureus isolates, suggesting that the activity of MecA cannot be distinguished from that of existing penicillin binding proteins (PBP), or that it is not functioning in cell wall biosynthesis, as long as these strains are cultivated in the absence of β-lactam antibiotics (de Jonge et al., 1992; Labischinski and Maidhof, 1994). When β-lactam antibiotics saturate the normal set of staphylococcal PBP, the cell wall is still produced due to the presence of MecA but is drastically hypcrosslinked. The expression of methicillin resistance does depend on fem genes (Berger-Bächi, 1994). The inactivation of femA and femB results in loss of resistance of MRSA strains and induction of hypersensitivity of sensitive staphylococci.

**ACQUISITION OF mecA AND ITS REGULATION**

Both annotated S. aureus N315 and Mu50 genomes contain a so-called “staphylococcal cassette chromosome mec” (SCCmec) which encodes resistance to β-lactams, bleomycin, macrolide-lincosamide streptogramin B, aminoglycosides (tobramycin and amikacin), and spectinomycin (Kuroda et al., 2001). One can also consider the 40–60-kb long SCCmec as a resistance island, which was acquired very likely by horizontal gene transfer.

In opposite orientation to meca are two co-transcribed genes, mecR1 and mecI. The mecR1 gene encodes a membrane-bound signal transduction protein (MecR1), while mecI encodes a transcriptional regulator (MecI). Between meca and mecR1 are the promoters for these genes and an operator region that encompasses the −10 sequence of meca and the −35 sequence of mecR1 (Sharma et al., 1998). MecR1 and MecI have high protein sequence homology with the proteins, BlaR1 and BlaI, respectively, that are involved in the inducible expression of the plasmid-mediated staphylococcal β-lactamase gene, blaZ. MecI is a tight regulator of meca transcription, and most β-lactam antibiotics do not efficiently activate MecR1 (Kuwahara-Arai et al., 1996). Consequently, some isolates, referred to as “pre-MRSA,” are methicillin-sensitive despite carrying the meca gene. However, selective pressure through antibiotic usage has promoted S. aureus isolates that have mutations or deletions in mecI or the meca promoter/operator region, giving rise to an inactive repressor and constitutive meca expression. These mutants can display homogeneous (all cells are resistant to high concentrations of methicillin [>128 μg/liter]) or heterogeneous (only a small minority of cells exhibit high-level methicillin resistance) methicillin resistance phenotypes (Kondo et al., 2001).

**Other Cell Wall Components**

**CELL WALL TEOIC ACID**

The staphylococcal cell wall consists not only of a thick peptidoglycan fabric but also of polymers of alternating phosphate and alditol groups called “teichoic acids.” The discovery of the teichoic acids resulted from the identification of ribitol in a cytidine nucleotide isolated and characterized by Baddiley and coworkers already in the mid-1950s. The size of ribitol teichoic acids and the nature of their linkage to glycosaminopolypeptides was described in 1965 (Hay et al., 1965).

There are two teichoic acid types in staphylococci: a wall teichoic acid (WTA), which is covalently bound to the peptidoglycan and which is, depending on the species, composed of ribitol and/or glycerol teichoic acids (Pooley and Karamata, 1994). The WTA is distinct from the subsequently discovered lipoteichoic acids (LTAs). LTA contains only glycerol phosphate and is anchored in the cytoplasmic membranes of Gram-positive bacteria (Fischer, 1988). The alditol units of WTAs and the LTAs contain normal substituents of which D-alanine and glycosyl residues are most common. The structures of the S. aureus WTA and LTA are shown in Fig. 11.

**STRUCTURE AND BIOSYNTHESIS OF WALL TEOIC ACID**

The cell wall teichoic acid structures of 13 staphylococcal type strains were determined (Endl et al., 1983). Most of the strains contain a poly(polyphosphate) teichoic acid with glycerol and/or ribitol as polyol component. The polyphosphate backbone is partially substituted with various combinations of sugars and/or amino sugars. Most of the substituents occur in a monomeric form, but some strains also contain dimers of N-acetylglucosamine as substitutents. *Staphylococcus hyicus* subsp. *hyicus* and S. sciuri revealed rather complex cell wall teichoic acids. They consist of repeating sequences of phos-
phate-glycerol-phosphate-N-acetylglucosamine. The amino sugar component is present in this case as a monomer or an oligomer. Moreover, the glycerol residues are partially substituted with N-acetylglucosamine. The cell wall teichoic acid of Staphylococcus auricularis is a poly(N-acetylglucosaminyl-phosphate) polymer similar to that found in *Macrococcus caseolyticus*.

Biosynthesis of ribitol teichoic acids was studied using membrane fractions of *S. aureus* H (Yokoyama et al., 1986). Incubation of *S. aureus* membranes with CDP-glycerol and ManNAc-[³¹⁴C]GlcNAc-PP-prenol led to synthesis of (glycerol phosphate)-1-3-ManNAc-[³¹⁴C]GlcNAc-PP-prenol. In *S. aureus* (glycerol phosphate)-2-ManNAc-[³¹⁴C]GlcNAc-PP-prenol as well as (glycerol phosphate)-3-ManNAc-[³¹⁴C]GlcNAc-PP-prenol served as an acceptor for ribitol phosphate units, but (glycerol phosphate)-ManNAc-[³¹⁴C]GlcNAc-PP-prenol did not. The covalent attachment of WTA to peptidoglycan does not appear to play a role in the maintenance of the shape or rigidity of the cell wall, since their extraction with trichloracetic acid (TCA) leaves an essentially intact residual wall structure (Baddeley, 1972). While the cell-biological functions of LTA are manifold, not much is known about WTA, which however plays an important role as bacteriophage receptor (Schleifer and Steber, 1974). It was for a long time an open question whether WTA is essential for growth. Recently in the group of A. Peschel, a completely WTA negative deletion mutant was isolated which exhibits normal growth but became resistant to a number of bacteriophages was impaired in adherence to nasal cells, and was completely unable to colonize cotton rat nares (Weidenmaier et al., 2004).

**LIPOTEICHOIC ACID (LTA)** With a pure (>99%) LTA preparation from *S. aureus*, nuclear magnetic resonance and mass spectrometry analyses were carried out (Morath et al., 2001). The results essentially corroborate the structure described by Fischer (1994). The average chain length of polyglycerol phosphate was 45–50 units; 70% of the glycerophosphate units were esterified with D-alanine, 15% bore α-D-N-acetylglucosamine, and 15% had no substituents. The fatty acids had an average chain length of C₁₅H₃₁. Fischer found that the alanine esters are rapidly lost from completed LTA and he hypo-
esized that by spontaneous hydrolysis of the labile ester bond, D-alanine is transferred to the WTA. The D-alanine loss in LTA is compensated by incorporation of new alanine ester at a rate adjusted to the velocity of loss (Fischer, 1994).

**Biological Functions** It has been proposed (Heptinstall et al., 1970) that teichoic acids (which are highly negatively charged polymers) play an important role in divalent cation sequestration at the bacterial surface. Indeed it was found that the alanine ester content influences the magnesium binding capacity of walls of *S. aureus* H grown at different pH values (Archibald et al., 1973).

Fischer and coworkers investigated the influence of the degree of D-alanine esterification of staphylococcal LTA on autolysin activity using extracellular autolysin from *S. aureus* (Fischer et al., 1981). It turned out that the inhibitory activity was highest with D-alanine-free LTA. Glycosylation of LTA up to an extent of 0.5 did not depress inhibitory activity. They hypothesized that the anti-autolytic activity of LTA resides in a sequence of glycerophosphate units and that the negative charges of appropriately spaced phosphodiester groups play a crucial role. This work shows that autolysis is regulated in vivo by the alanine ester content of the LTA, and very likely by the WTA too, which also carries ester bound D-alanine. LTA is also an important antigen (Knox and Wicken, 1973). Some of the biological properties of the staphylococcal envelope components are listed in Table 13.

Apart from lethal toxicity and pyrogenicity, LTA appeared to share many of the biological properties exhibited by lipopolysaccharides (Wicken and Knox, 1980). Critical micelle concentrations of various LTAs has been determined to be 1–10 μg/liter, suggesting that acylated LTAs in their monomer forms may represent the major configuration of extracellular LTAs in bacterial culture fluids (Wicken et al., 1986).

Great care has to be taken when analyzing commercial LTA preparations for immune stimulating activity. One must be aware that frequently these preparations are inhomogeneous and contain decomposed LTA and endotoxins of more than 10 ng of LPS/mg of LTA (Morath et al., 2002). A novel isolation procedure has been worked out that leads to a pure (> 99%) biologically active LTA, allowing the first structural analysis by nuclear magnetic resonance and mass spectrometry. A comparison with LTA purified by standard techniques revealed that alanine substituents are lost during standard purification, resulting in attenuated cytokine induction activity. In line with this finding, hydrolysis of alanine substituents of active LTA eliminated cytokine induction. Purified *S. aureus* LTA induced the release of TNF-α, IL-1β, IL-6 and IL-10 in human whole blood. Soluble CD14 (sCD14) inhibited monokine induction by LTA but failed to confer LTA responsiveness for IL-6 and IL-8 release of human umbilical vein endothelial cells (HUVECs). In a competitive LPS-binding protein (LBP) binding assay, the IC₅₀ of the tested LTA preparations was up to 3230-fold higher than for LPS. The LBP enhanced TNF-α release of human peripheral blood mononuclear cells (PBMCs) upon LPS but not LTA stimulation. These data demonstrate a differential role for the serum proteins LBP and sCD14 in the recognition of LPS and LTA (Hermann et al., 2002). There is evidence that the recognition sites of CD14 for LPS and LTA are distinct with a partial overlap. While the maximal achievable monokine release in response to LTA or to LPS was comparable, all LTA induced significantly less IL-12 and IFN-γ. IL-12 substitution increased LTA-inducible IFN-γ release up to 180-fold, suggesting a critical role of poor LTA-inducible IL-12 for IFN-γ formation. Pretreatment with IFN-γ rendered galactosamine-sensitized mice sensitive to challenge with LTA. When compared to LPS, LTA (a major immunostimulatory component of *S. aureus*) is a weak inducer of IL-12 and subsequent IFN-γ formation, which might explain the lower toxicity in vivo.

In a recent study, the effects of LPS from *E. coli*, LTA and peptidoglycan (PG) from *S. aureus* and live *S. aureus* on leukocyte-endothelial interactions in vivo have been investigated (Yipp et

Table 13. Some biological properties of staphylococcal envelope components.

| Envelope components | Property or activity |
|---------------------|---------------------|
| Peptidoglycan       | Cell shape          |
|                     | Osmotic stability   |
|                     | Antigenic           |
|                     | Pyrogenic           |
| Peptidoglycolipid   | Adjuvant            |
| Muramylpeptides     | Somnogenic          |
| WTA                 | Antigenic           |
|                     | Phage receptor      |
|                     | Divalent cation binding |
| LTA                 | Autolysis inhibition/regulation |
|                     | Immunogenic         |
|                     | Cell adhesion       |
|                     | Phage receptor      |
|                     | Complement activation |
|                     | Autolysis inhibition/regulation |
| Lipoprotein         | Cell binding        |
|                     | Immunogenic         |
|                     | Mitogenic           |

Abbreviations: WTA, wall teichoic acid; and LTA, lipoteichoic acid.

*From Salton (1994).*
al., 2002). It was found that local administration of LPS into muscle induced significant leukocyte rolling, adhesion, and emigration in postcapillary venules at the site of injection. Given systemically, LPS caused circulating leukocyte counts to drop dramatically and neutrophil counts to increase in the lung. However, the drop in circulating leukocytes was not associated with leukocyte sequestration to the site of injection (peritoneum) or to peripheral microvessels in muscles. Unlike LPS, LTA had no systemic and very minor local effect on leukocyte-endothelium interactions, even at high doses and prolonged exposure. LPS, but not LTA, potently activated human endothelium to recruit leukocytes under flow conditions in vitro. Endothelial adhesion molecule expression was also increased extensively with LPS, but not LTA. Interestingly, systemic administration of live *S. aureus* induced leukocyte-endothelial cell responses similar to LPS. PG was able to induce leukocyte-endothelial interactions in muscle and peritoneum, but had no effect systemically (no increase in neutrophils in lungs and no decrease in circulating neutrophil counts). These results demonstrate that: 1) LPS has potent, but divergent local and systemic effects on leukocyte-endothelial interactions; and 2) *S. aureus* can induce a systemic response similar to LPS, but this response is unlikely to be due to LTA, but more likely to be mediated in part by PG.

Toll-like receptors (TLRs) are involved in cellular activation by microbial products, including lipopolysaccharide, lipoproteins, and peptidoglycan. Although for these ligands the specific transmembrane signal transducers TLR-4, TLR-2, or TLR-2 and -6 have now been identified, the molecular basis of recognition of LTA and related glycolipids has not been completely understood. Purified LTAs from *S. aureus* and *B. subtilis* exhibited TLR-2 dependence in nuclear factor kB activation and cytokine induction. The signaling molecules MyD88 and NIK appear to be also involved in cell stimulation by LTA (Opitz et al., 2001). The results presented here suggest that TLR-2 is the main receptor for the LTA-mediated inflammatory response.

Alanine Esterification and Virulence *Staphylococcus aureus* and *S. xylosus* normally tolerate high concentrations of several positively charged antimicrobial peptides. Andreas Peschel isolated staphylolococcal transposon mutants that were not only hypersensitive to lantibiotics such as gallidermin (Göttz and Jung, 2001a) but also to human defensin HNP1-3, animal-derived protegrins, and other antimicrobial peptides (Peschel et al., 1999). All these substances are cationic antimicrobial peptides (CAMPs), many of which have membrane-damaging activity.

One mutant type lacked ester-bound D-alanine in the teichoic acids of both WTA and LTA. As a result the cells carry an increased negative surface charge and bound fewer anionic, but more positively charged peptides and proteins (Peschel et al., 1999). In addition, they became much more sensitive to human defensins, animal-derived protegrins, tachyplesins, and magainin II, and to the lantibiotics gallidermin and nisin. The mutation was located within the *dlt* genes involved in the transfer of D-alanine into teichoic acids. The fact that high concentrations of magnesium ions compromised the protective properties of D-alanine esters corroborates the hypothesis that the increased negative charge of the cell wall is responsible for the hypersensitivity against positively charged antimicrobial peptides. These results also show that the D-alanine-esterification of teichoic acids (which occurs in many Gram-positive bacteria) is not essential for growth but is essential in the protection against human and animal defense systems (Peschel et al., 1999). The innate immune system is largely based on the release of defensins by immune cells and various tissue cells such as epithelial or endothelial cells. Therefore, *dltA* mutants should be less virulent than wild type cells. Indeed it was demonstrated that the *S. aureus* *dltA* mutant lacking D-alanine modifications of teichoic acids is highly susceptible to neutrophil killing and is virulence-attenuated in mice (Collins et al., 2002). These results clearly show that D-alanine-esterification of teichoic acids is a virulence factor.

Substances such as defensins from the granules of phagocytes, epithelial surfaces, and skin (Ganz and Lehrer, 1994) share an amphiphilic cationic structure and a membrane-damaging activity by forming pores or disintegrating the cytoplasmic membrane bilayer. They are not only produced by mammals but also by insects and bacteria (Sahl and Bierbaum, 1998). CAMPs represent an important human defense mechanism, protecting skin and epithelia against invading microorganisms and assisting neutrophils and platelets. *Staphylococcus aureus* and other bacterial pathogens have evolved countermeasures to limit the effectiveness of CAMPs, including the repulsion of CAMPs by reducing the net negative charge of the bacterial cell envelope through covalent modification of anionic molecules (e.g., teichoic acids, phospholipids and lipid A). Mutants susceptible to CAMPs are more efficiently inactivated by phagocytes and are virulence-attenuated, indicating that CAMP resistance plays a key role in bacterial infections as reviewed by Peschel (2002).

Another biological effect of the *S. aureus* *dltA* mutant was that it became biofilm-negative. The underlying mechanism is its attenuated adher-
ence to artificial surfaces that play a key role in the first step of biofilm formation. The lack of D-alanine esters with the associated increase of negative surface charge affected colonization of polystyrene or glass (Gross et al., 2001), while PIA (polysaccharide intercellular adhesion) production, necessary for accumulated growth was only marginally influenced in the mutant. The data suggest that repulsive electrostatic forces can lead to reduced staphylococcal biofilm formation. Interestingly, the addition of >10 mM MgCl₂ but not CaCl₂ completely restored biofilm formation, again corroborating the important role of teichoic acids in scavenging Mg²⁺ ions.

### Cell Wall Bound Proteins

Many surface proteins of Gram-positive bacteria are anchored to the cell wall envelope by a transpeptidation mechanism, requiring a C-terminal sorting signal with a conserved LPXTG motif (leucine-proline-unknown-threonine-glycine). Pioneering work in the identification and characterization of the enzyme involved in protein anchoring and the underlying mechanism was carried out in the group of Olaf Schneewind. In a recent review, the main achievements are summarized (Mazmanian et al., 2001).

**Protein A — A Cell Wall-anchored Surface Protein**

Protein A, an immunoglobulin G (IgG) binding protein, was the first identified surface protein of *S. aureus* (Sjödahl, 1977). Protein A is covalently bound via its C-terminal end to the pentaglycine interpeptide bridge of peptidoglycans and can be released by treatment with lysostaphin, a glycol-glycine endopeptidase. Protein A (Spa) was one of the first proteins shown to be covalently anchored to the peptidoglycan. Protein A is synthesized as a precursor, containing an N-terminal signal peptide, a pro-peptide region, five IgG-binding domains (Moks et al., 1986), a cell-wall spanning region, and finally a 35-residue cell wall sorting (cws) sequence which is conserved in surface anchored proteins of most Gram-positive bacteria (Schneewind et al., 1992). The cws consists of the typical LPXTG motif, which is followed by a hydrophobic domain and a tail of mostly positively charged residues. Mutations at the LPXTG motif lead normally to the secretion of the mutant protein into the extracellular medium. Deletion of this motif prevents cleavage and cell wall anchoring of protein A. The cws sequence of protein A is representative (Fig. 12).

Apart from *spa*, there are in *S. aureus* several other proteins with a typical cws sequence (Table 14).

**SORTASE Sortase (SrtA)**

SORTASE Sortase (SrtA) cleaves polypeptides between the threonine and the glycine of the LPXTG motif (peptidase reaction) and catalyzes the formation of an amide bond between the carboxyl-group of threonine and the amino group of peptidoglycan crossbridges (transpeptidation reaction; Mazmanian et al., 2001). Even if the pentaglycine anchor structure of the cell wall is altered by incorporation of serine residues, anchoring to the cell wall is not affected (Strauss et al., 1998).

**Staphylococcus aureus** mutants lacking the *srtA* gene fail to anchor and display some surface proteins (mostly protein A and fibronectin-binding protein) and are impaired in the ability to cause animal infections (Mazmanian et al.,

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**Table 14. Staphylococcus aureus** surface proteins.

| Protein | Length (aa) | Ligand | References |
|---------|-------------|--------|------------|
| Spa     | 508         | IgG, vWBF | Uhlen et al., 1984 |
| FnbA    | 1018        | Fibronectin or fibrinogen | Signäs et al., 1989 |
| FnbB    | 914         | Fibronectin or fibrinogen | Jönsson et al., 1991 |
| ClaA    | 933         | Fibrinogen | McDevitt et al., 1994 |
| ClaB    | 913         | Fibrinogen | Ni Eidhin et al., 1998 |
| Cna     | 1183        | Collagen | Patti et al., 1994 |
| SdrC    | 947         | Unknown | Josefsson et al., 1998a |
| SdrD    | 1315        | Calcium | Josefsson et al., 1998b |
| SdrE    | 1166        | Bone sialoglycoprotein | Tung et al., 2000 |
| Pls     | 1637        | None* | Savolainen et al., 2001 |
| SasA to SasJ | ND   | Unknown | Genome sequence |

Abbreviation: aa, number of amino acids; vWBF, von Willebrand-Factor; Sas, *S. aureus* surface proteins; and ND, not determined.

*Inhibits adhesion to fibronectin or fibrinogen.

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**Fig. 12.** Cell wall sorting (cws) sequence of protein A.
Sortase acts on surface proteins that are normally secreted by the classical secretory “sec” pathway and whose signal peptide is removed by the signal peptidase.

Recently, a second sortase gene (srtB) was identified. SrtB appears to be required for anchoring of a surface protein with an asparagine-proline-glutamine-threonine-asparagine (NPQTN) motif. An srtB mutant is defective in the persistence of animal infections. The srtB gene is part of an iron-regulated locus called “iron-responsive surface determinants” (isd), which also contains a ferrichrome transporter and surface proteins with NPQTN and LPXTG motifs. It is suggested that SrtB might be involved in iron acquisition (Mazmanian et al., 2002).

When tethered to the C-terminus of other proteins bearing N-terminal signal peptides, the cws promotes anchoring of hybrid protein to the cell wall (Schneewind et al., 1993; Navarre et al., 1996). It was also possible to anchor enzymes such as lipases or β-lactamases in active form to the staphylococcal cell wall, provided that a cell wall spanning region is upstream of the LPXTG motif (Strauss and Götz, 1996).

Slime and Biofilm Formation

The molecular and biochemical basis of biofilm formation in *S. aureus* and *S. epidermidis* has been recently reviewed (Götz, 2002). The first reports that slime-producing *S. epidermidis* strains are involved in catheter-associated sepsis and infections came from the groups of Peters (Peters et al., 1981), Christensen (Christensen et al., 1982), and Costerton (Marrie et al., 1982b). Mostly *S. epidermidis*, but sometimes also mixed cultures have been isolated from colonized catheters recovered from patients. The bacteria appeared to be closely packed and cemented together by a slimy matrix (Peters et al., 1981). Transmission and scanning electron micrographs showed again that *S. epidermidis* forms a confluent biofilm, in which the cells are embedded in an amorphous material referred to as “slime” (Christensen et al., 1982). These early observations and pioneering work indicated that 1) the skin bacterium *S. epidermidis*, once considered to be harmless, is an opportunistic pathogen that could cause chronic staphylococcal infection and 2) in certain strains, slime (an extracellular polysaccharide) is produced and contributes to the formation of a confluent biofilm. As summarized recently, *S. epidermidis* can form a biofilm on nearly any synthetic polymers used as prosthetic devices, and in addition, they can bind to blood proteins, matrix proteins, and human cell receptors (Götz and Peters, 2000a; Götz et al., 2000b).

Today *S. epidermidis* is regarded as a leading species in causing chronic polymer-associated clinical infection. Microscopy of biofilm formation in vitro suggests that two steps are involved: 1) the attachment of the bacterial cells to the polymer surface, and 2) the growth-dependent accumulation to form multilayered cell clusters surrounded by a slimy matrix (Fig. 13).

The slime consists of a polysaccharide composed of β-1,6-linked N-acetylglucosamine, 15–20% of which is de-acetylated and therefore positively charged (Mack et al., 1996). Also identified was another polysaccharide II (<20%), which is structurally related, but has a lower content of non-N-acetylated D-glucosaminyl residues and contains phosphate and ester-linked succinate, rendering it anionic. The structure of this polysaccharide is unique, and according

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**Fig. 13.** Two-step model of staphylococcal biofilm formation. The first step is the adherence of the bacterial cells to a surface. The second is the embedding of the cells in a thick slime matrix (biofilm). Within the biofilm, cells appear to have reduced physiological activity in an anoxic environment, and exhibit a decreased sensitivity to many antibiotics, compared with their planktonic counterparts. See also Götz and Peters (2000a).
to its function in intercellular aggregation, it was referred to as “polysaccharide intercellular adhesin” (PIA; Mack et al., 1996). A recently described polysaccharide PNSG (poly-N-succinyl β-1-6 glucosamine; McKenney et al., 1999) from *S. aureus* MN8N, containing N-acetylglucosamine residues regarded as completely succinylated, turned out to be an artefact (G. Pier, personal communication). PIA has several functions (Götz, 2002): it is a major factor of biofilm formation, contributes to the pathogenesis of biomaterial-associated infections (Rupp et al., 2001), is immunogenic, is involved in hemagglutination, binds to hydrophilic surfaces, and finally causes intercellular adhesion. Detection methods for PIA and biofilm formation have been recently described (Cramton et al., 2000; Leriche et al., 2000).

**Polysaccharide Intercellular Adhesion**  
Biosynthesis  
The genes responsible for biofilm formation were identified by isolating and analyzing biofilm-negative transposon-insertion mutants of *S. epidermidis* O-47 (Heilmann et al., 1996a). In this way, a number of genes could be identified which are involved in biofilm formation. There is the *ica* (intercellular adhesion) operon, which is responsible for the biosynthesis of PIA and its transcriptional control (Heilmann et al., 1996b). The operon is composed of the *icaR* (regulatory) gene and *icaADBC* (biosynthesis) genes. The in vitro biosynthesis of PIA has been analyzed using the membrane fraction or cellular or extracellular extracts (Gerke et al., 1998). IcaA has N-acetylglucosaminyltransferase activity with UDP-N-acetylglucosamine as substrate. IcaD might be a chaperone that directs the correct folding and membrane insertion of IcaA and, in addition, might act as a link between IcaA and IcaC. The role of IcaC is still unclear.

Other biofilm-negative staphylococcal mutants were defective in adherence to polymers and extracellular matrix. The deletion of the major autolysin *atlE* gene revealed a pleiotropic effect (Heilmann et al., 1997): Because of their amidase deficiency, the mutant cells form huge cell clusters that resist even detergent treatment, which points to a defect in cell separation. Owing to extracellular processing of the precursor AtlE, five surface-bound proteins were missing. Binding to vitronectin and fibronectin was reduced, and finally, adherence to and biofilm-formation on hydrophobic surfaces was markedly decreased.

In a search for *S. aureus* mutants hypersensitive to positively charged antimicrobial peptides, mutants with an altered teichoic acid structure have been isolated (Peschel et al., 1999). The mutations are in the *dltA* operon, and the teichoic acids lack D-alanine. It has been postulated that the increased negative charge of the cell surface of the mutants leads to an increased scavenging of positively charged antimicrobial peptides, thus leading to the observed hypersensitivity. The *dltA* mutant is also biofilm-negative even though PIA production appears to be unchanged. The mutant is severely affected in adherence to polystyrene or glass surfaces (Gross et al., 2001), the first step of biofilm formation. In this respect, the *dltA* mutant resembles the *atlE* mutant described above, with one exception: the *atlE* mutant is still able to form a biofilm on a glass surface. That biofilm formation of the *dltA* mutant can be completely restored by the addition of Mg^{2+} ions corroborates the biological importance of the charge balance of the Gram-positive cell surface.

Other proteins can also contribute to biofilm formation in staphylococci, such as the accumulation-associated protein (AAP; Hussain et al., 1997) and the biofilm-associated protein (Bap; Cucarella et al., 2001). Especially *S. aureus* is capable of adhering to a large variety of matrix components to initiate colonization. This adherence is frequently mediated by protein adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, which in many cases are covalently anchored to the cell wall peptidoglycan (Foster and Hööck, 1998). Very likely, surface proteins such as fibronectin-binding protein A and B (FnBPA and FnBPB), the collagen-binding protein Cna, and the fibrinogen-binding proteins, clumping factor A and B (ClfA and ClfB), contribute to adherence and thus to biofilm formation.

Slime production and biofilm formation are regulated by a number of environmental parameters and stress factors, which were summarized by Götz (2002). Among them, anaerobiosis clearly induces ica expression (Cramton et al., 2001). It was also shown that IS256 integrates into the ica operon and can also be precisely excised (Ziebuhr et al., 1999; Ziebuhr et al., 2000a; Ziebuhr et al., 2000b). The role of PIA in pathogenicity is also corroborated by the finding that an intact ica operon is more prevalent in clinical (septicemic disease and shunt-associated meningitis) *S. epidermidis* isolates than in skin isolates of nonhospitalized persons (Ziebuhr et al., 1997).

Biofilm formation in staphylococci is multifactorial, and the ability to form a biofilm makes the strains much better able to survive in the normally hostile environment of tissue and blood. Biofilm formation appears to be a bacterial survival strategy that is turned on when, for example, oxygen and Fe ions become limited, when sublethal concentrations of certain antibiotics are present, or other stress
situations emerge. The modulation of biofilm formation by various environmental conditions appears to be an advantage for successful infection.

**New Membrane Components** Another mutant type was hypersensitive to CAMPs (Peschel et al., 2001) and identified with a novel staphylococcal gene, *mprF*. The *mprF* mutant strain was killed considerably faster by human neutrophils and exhibited attenuated virulence in mice, indicating a key role for defensin resistance in the pathogenicity of *S. aureus*. In the *mprF* mutant, the predominant membrane component phosphatidylglycerol lacks L-lysine modification. The structure of lysophosphatidylglycerol (L-PG) is shown in Fig. 14. As this unusual modification leads to a reduced negative charge of the membrane surface, MprF-mediated peptide resistance is most likely based on repulsion of the cationic peptides. Accordingly, inactivation of *mprF* led to increased binding of antimicrobial peptides by the bacteria. The *mprF* gene has no similarity with genes of known function, but related genes were identified in the genomes of several pathogens including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. MprF thus constitutes a novel virulence factor, which may be of general relevance for bacterial pathogens and represents a new target for attacking multidrug resistant bacteria.

**Protein Secretion in Staphylococci**

The *S. aureus* genome was screened for secretion genes of the classical sec pathway (Kuroda et al., 2001; Mazmanian et al., 2001). Apart from the *E. coli* specific secB and secF genes, all other genes (secA, D, E, G, Y; yajC; fliC; ftsY, and lepB) are also present in the *S. aureus* genome. In addition, the *S. aureus* genomes revealed two secA (secA-1 and secA-2) and two secY genes (secY-1 and secY-2). The presence of two homologous genes is unclear; however, one can speculate that they play a role for a specific set of translocated proteins, like for example *srtB*, whose function differs apparently from that of *srtA*. The presence of most of the sec genes suggests that in *S. aureus*, the sec pathway is essentially similar to that described for *E. coli*. Maybe this is the reason why protein secretion is hardly studied in staphylococci. However, it was shown that many of the secreted proteins in staphylococci possess a so-called “pro-peptide region” and the role of this region in protein expression and secretion has been studied in more detail.

**Role of the Pro-peptide Region in Exoproteins** Since the *S. hycicus* specific lipase (SHL) was the first bacterial lipase gene ever to be cloned and sequenced (Göttz et al., 1985), both the enzymatic properties and the role of the pro-peptide was investigated in more detail with this enzyme (reviewed in Götz and Rosenstein, 2001b).

According to sequence comparisons, all staphylococcal lipases are predicted to be primarily pre-pro-lipases. A structural organization of some of the lipases is shown in Fig. 15. The various pro-peptides have no striking similarities at the sequence level but are distinguished by

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Fig. 14. Structure of lysophosphatidylglycerol (L-PG).

Fig. 15. Organization of staphylococcal (phospho)lipases as pre-pro-enzymes. SHL, (phospho)lipase from *S. hycicus* subsp. *hycicus* DSM 20459; SAL-2, lipase from *S. aureus* NCTC 8530; SAL-1, lipase from *S. aureus* PS54; SEL-2, lipase from *S. epidermidis* RP62A. sp, signal peptide; and pp, pro-peptide region. The proposed active site amino acids are indicated in the mature form of each enzyme. aa, amino acid number or position; and IEP, isoelectric point. From Götz et al. (1998).
their overall hydrophilic character. To get more insight into the function of the pro-peptide, internal deletions within this region were made and analyzed (Demleitner and Götz, 1994). The results obtained with these lipase mutants indicate that the SHL pro-peptide may have two functional domains, with each one located in one half of the pro-region. The N-terminal part seems to be important for lipase activity and the C-terminal part, for translocation and stability. A stabilizing effect of the SHL pro-peptide has also been observed in an experiment where OmpA of E. coli is fused to the pre-pro portion of SHL; without the SHL pro-peptide, massive proteolytic degradation occurred after secretion by S. carnosus (Meens et al., 1997). A number of experiments designed to address the question whether the pro-peptide could act also in trans indicated that it has no trans activity. It exerts its beneficial effects on translocation, stability, and activity only intramolecularly (G. Thumm et al., unpublished observation). All studies carried with the pro-peptide speak in favor of an intramolecular chaperon-like function. Maybe it compensates in Staphylococcus and also in Bacillus for the lack of the SecB homolog.

The proteolytic processing of pro-SHL has been studied in more detail (Liebl and Götz, 1986; Götz, 1991; Demleitner and Götz, 1994). Since the 86-kDa pro-SHL purified from S. carnosus is processed by culture supernatants of S. hyicus, it became apparent that the processing of the pro-region by an extracellular protease occurs after secretion. The proteolytic activities in the supernatant of S. hyicus were analyzed, and two proteases (ShpI and ShpII) were identified; ShpII proved to be involved in pro-SHL processing (Ayora and Götz, 1994a; Ayora et al., 1994b).

### The Use of SHL as Expression and Secretion System
The beneficial role of the pro-peptide region of SHL for efficient secretion of heterologous proteins and enzymes was already described earlier (Liebl and Götz, 1986). The results support a dual role for the SHL pro-peptide: an involvement in protein translocation and a role in stabilization against proteolytic degradation.

Because of these encouraging results, the pre-pro-portion of SHL was fused to several other heterologous proteins such as human growth hormone (Sturmfels et al., 2001) and malaria antigen (Samuelson et al., 1995). In all cases, the heterologous proteins were successfully and in good quantities secreted by S. carnosus, indicating a general application of this strategy.

An alignment of the lipase signal peptides shows a remarkable motif containing the perfectly conserved residues Ser, Ile, Arg and Lys, designated as the “SIRK-motif” (Götz et al., 1985; Nikoleit et al., 1995). This motif is present in many exoproteins of Gram-positive bacteria. Its function is still unclear.

### Genomic Analysis of Virulence Genes
Several staphylococcal strains are completely sequenced but so far only the S. aureus strains N315 and Mu50 (Kuroda et al., 2001) are annotated and permit a general protein search. Some general features of S. aureus N315 genome are listed in Table 15.

To see whether and to which extent the genomes of S. aureus N315 (causing acute infections) and S. epidermidis RP62A (causing chronic infections) differ from each other, an arbitrary list of 125 virulence or fitness genes of the S. aureus N315 genome were investigated for the presence of homologous genes in S. epidermidis RP62A (Nerz et al., 2002). Genes or operons were selected that are involved in the production of exoenzyme, toxins, adhesins including biofilm formation, Fe-uptake, resistance to various antibiotics, and other functions. The comparison of both genomes was complicated by the fact that the RP62A sequence was

| Chromosome | Length of sequence (bp) | 2,813,641 |
|------------|------------------------|-----------|
| G+C content (total genome) | 32.8% |
| ORFs Percentage coding | 84.5% |
| Protein coding regions | 2595 |
| Ribosomal RNAs | | |
| 16S | 5 |
| 23S | 5 |
| 5S | 6 |
| t-RNAs | 62 |
| tmRNAs | 1 |
| Insertion sequences | | |
| IS181 | 8 |
| IS431 | 2 |
| Others | 10 |
| Transposons | | |
| Tn554 | 5 |
| Others | 0 |
| Bacteriophages | 1 |
| SCCmec | 1 |
| **Pathogenicity islands** | 3 |
| Plasmid | | |
| Length of sequence (bp) | 2465 |

Abbreviations: tmRNA, RNA that has dual tRNA-like and mRNA-like nature; IS, insertion sequence; Tn, transposon; SCCmec, staphylococcal cassette chromosome mec; and mec, methicillin-resistance component. From Kuroda et al. (2001).
not yet annotated. The selected virulence genes were localized on the \textit{S. aureus} N315 genome (at the DOGAN-Server) by their ID-number and translated into amino acid sequence. The protein sequences were used as queries in a tBLASTn search of the \textit{S. epidermidis} RP62A genome at The Institute for Genomic Research (TIGR) Web site. We also checked the almost finished genome sequence of the food-grade \textit{Staphylococcus carnosus} TM300 (R. Rosenberg, C. Néry, and F. Götz). To evaluate whether the resulting matches could be regarded as significant and thus represent homologous genes, they were checked carefully for length of alignment, percentage of similarity/identity and presence of functional protein domains. For this analysis, we used the following Web sites/tools:

In Tables 16 to 20, the genes identified in \textit{S. aureus} N315 and the corresponding protein names (if available) are indicated. Those genes that have homologous counterparts in \textit{S. carnosus} TM300 are highlighted in red.

Of the 159 selected virulence genes in \textit{S. aureus} N315, only 37 genes (18\%) had a homologous equivalent in \textit{S. epidermidis} RP62A and 31 genes had homologous equivalent in \textit{S. carnosus} TM300 (Rosenstein, Néry, and Götz). This clearly reflects the difference in the pathogenic potential of the three species representatives and explains why \textit{S. aureus} is rather an aggressive pathogen, \textit{S. epidermidis} a mild pathogen and \textit{S. carnosus} is non-pathogenic. Of the 40 toxin genes in \textit{S. aureus}, only 3 and 5, respectively, were identified in the \textit{S. epidermidis} and \textit{S. carnosus} genome. This finding is in agreement with the earlier observations of decreased toxin production of \textit{S. epidermidis} and corroborates well with the decreased severity of an \textit{S. epidermidis}

| Exoenzymes | ORFs | Gene name | Function | Similarity (%) |
|------------|------|-----------|----------|---------------|
| Staphylococcal clumping | SA0222 | coa | Possible coagulation in tissues | — |
| Possible staphylococcal clumping | SA0743 | None | Unknown | — |
| Thermonase | SA1160 | nuc | Degradation of host’s nucleic acid | 77 |
| Staphylococcal nucleosome | SA0746 | nuc | Degradation of host’s nucleic acid | — |
| Probable 59-nucleotidase | SA0022 | None | Unknown | 62 |
| Lipase | SA0309 | geh | Hydrolytic degradation of lipids | — |
| Triacylglycerol lipase | SA2463 | lip | Hydrolytic degradation of lipids | 56 |
| Similar to lipase LipA | SA0610 | None | Unknown | 77 |
| Probable lipase | SA2323 | None | Unknown | — |
| 1-Phosphatidylinositol phosphodiesterase | SA0091 | plc | Hydrolysis of phosphatidylinositol | — |
| Hyaluronate lyase | SA2003 | hysA | Degradation of host’s hyaluronic acid | — |
| Serine protease<sup>a</sup> | SA0901 | sspA | Proteolytic destruction of host tissues | — |
| Serine protease | SA0879 | None | Proteolytic destruction of host tissues | — |
| Probable serine protease | SA1627, 1628, 1629, 1630, and 1631 | splA, splB, splC, splD, and splF | Unknown | — |
| Cysteine protease | SA0900 | sspB | Proteolytic destruction of host tissues | — |
| Staphopain<sup>b</sup> | SA1725 | None | Proteolytic destruction of host tissues | — |
| Zinc metalloproteinase aureolin | SA2430 | aur | Proteolysis of host tissues | — |
| Staphylokinase<sup>c</sup> | SA1758 | sak | Proteolytic destruction of host tissues | — |

Table 16. Presence of exoenzymes in \textit{S. aureus} N315/Mu50 and \textit{S. carnosus} TM300.

Symbol and abbreviations: —, not determined; ORFs, open reading frames; agr, accessory gene regulator; and Tnase, thermonuclease.
<sup>a</sup>EC-Number 3.1.31.1; typical for \textit{S. aureus}.
<sup>b</sup>Hyalurondidase.
<sup>c</sup>V8 protease.
<sup>d</sup>Cysteine protease.
<sup>e</sup>Protease III.
Blue indicates newly identified ORFs.
Red indicates counterpart in the \textit{S. carnosus} TM300 genome.
Table 17. Presence of toxins in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

| Toxins                  | ORFs              | Gene name | Function                              | Similarity (%) |
|-------------------------|-------------------|-----------|---------------------------------------|----------------|
| α-Hemolysin             | SA1007            | hla       | Destruction of blood and tissue cells (agr-controlled) | —              |
| γ-Hemolysin components  | SA2207, 2208, and 2209 | hlgA, hlgC, and hlgB | Destruction of blood cells | —              |
| θ-Hemolysin             | SAS065            | hld       | Destruction of blood and tissue cells (agr-controlled) | 77             |
| Leukotoxins             | SA1637 and 1638   | lukD and lukE | Destruction of white blood cells | —              |
| Possible leukocidin     | SA1813            | lukM      | Unknown                                 | —              |
| Possible hemolysin      | SA065             | None      | Unknown                                 | 89             |
| Possible hemolysin      | SA0780            | None      | Unknown                                 | 82             |
| Similar to exotoxin I   | SA1009            | None      | Unknown                                 | 55             |
| Possible hemolysin      | SA1812            | None      | Unknown                                 | —              |
| Similar to hemolysin III| SA1973            | None      | Unknown                                 | 75             |
| Possible exotoxins      | SA0357            | None      | Unknown                                 | —              |
|                        | SA0382, 0383, 0384| set6, set7, set8, set9 | Unknown                                | —              |
|                        | Location: SaPIm2/SaPIm3 |          |                                        |                |
|                        | 0385, 0386, 0387  | set10, set11, set12 |                                        | —              |
|                        | 0388, 0389, 0390, and 0393 | set13, set14 |                                        | —              |
|                        | SA1009, 1010, and 1011 |          |                                        | —              |
| Superantigens           | SA1642, 1643, 1644, 1645, 1646, 1647, and 1648 | seg, sen, ent1, ent2, ent3, sei, sem, and seo | Food poisoning, superantigen (agr-controlled) | —              |
| Enterotoxins            | SA1761(N315)      | sep       | Food poisoning, superantigen           | —              |
| Enterotoxin A           | SAV119(Mu50)      | sea       | Food poisoning, superantigen           | —              |
| Enterotoxins            | SA1816 and 1817   | sel and sec3 | Food poisoning, superantigen           | —              |
|                        | Location: SaPIm1/SaPIm1 |          |                                        |                |
| Toxic shock syndrome    | SA1819            | tst       | Fever, shock, skin rash, superantigen (agr-controlled) | —              |
|     toxin 1             | Location: SaPIm1/SaPIm1 |          |                                        |                |
| Probable enterotoxin    | SA1430            | None      | Unknown                                 | —              |

Symbol and abbreviation: —, not determined; and agr, accessory gene regulator.
Blue indicates newly identified ORFs.
Red indicates counterpart in the *S. carnosus* TM300 genome.

Table 18. Presence of adhesins in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

| Adhesins                  | ORFs              | Gene name | Function                              | Similarity (%) |
|---------------------------|-------------------|-----------|---------------------------------------|----------------|
| IgG-binding protein A     | SA0107            | spa       | Potential immune disorder in host (agr-controlled) | —              |
| IgG-binding protein SBI   | SA2206            | sbi       | Potential immune disorder in host      | —              |
| Ser-Asp-rich fibrinogen-binding proteins | SA0742 and 2423 | clfA, clfB | Cellular adhesion onto host tissues | —              |
| Possible fibrinogen-binding proteins | SA1000, 1003, and 1004 | None | Unknown | — |
| Fibronectin-binding proteins | SA2290 and 2291 | fnbB and fnbA | Cellular adhesion onto host tissues (agr-controlled) | — |
| Ser-Asp-rich proteins     | 0519, 0520 and 0521 | sdrC, sdrD and sdrE | Cellular adhesion onto host tissues | — |
| Possible extracellular matrix binding proteins | SA0744 and 0745 | | Cellular adhesion onto host tissues | — |
| Probable extracellular matrix binding proteins | SA1267 and 1268 | ebhA and ebhB | Unknown | — |
| Sortase A                 | SA2316            | srtA      | Covalent linkage of binding proteins to the peptidoglycan | 74%            |
| Sortase B                 | SA0982            | srtB      | Covalent linkage of binding Fe-protein to the peptidoglycan | —              |
Table 18.  Continued

| Adhesins | ORFs | Gene name | Function | Similarity (%) |
|----------|------|-----------|----------|----------------|
| Proteins with the cell wall sorting signals (LPXTG) | SA0976, 0977, 1552, 1577, 1888, 1964, 2284, 2381, and 2447 | None | Possible pathogenic factors | — |
| Probable adhesin | SA0857 | None | Cellular adhesion onto host tissues | 83 |
| Elastin-binding protein | SA1312 | ebpS | Cellular adhesion onto host tissues | — |

Symbol and abbreviations: −, not determined; IgG, immunoglobulin G; and LPXTG, leucine-proline-unknown-threonine-glycine.

*Similarity: 77% (ubiquitous).

Blue indicates newly identified ORFs.

Red indicates counterpart in the *S. carnosus* TM300 genome.

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Table 19. Presence of Fe-uptake and other virulence genes in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

| Fe-uptake | ORFs | Gene name | Function | Similarity (%) |
|-----------|------|-----------|----------|----------------|
| Possible siderophore biosynthesis proteins | SA0116 and 0117 | None | Iron uptake | — |
| Possible iron-binding protein | SA0217 | None | Iron uptake | — |
| Possible iron permease components | SA0566 and 0567 | None | Iron uptake | 73, 60 |
| Possible ferrichrome ABC transporter components | SA0891 | None | Iron uptake | 60 |
| Possible ferritin | SA1709 | None | Maintenance of cellular iron | — |
| Possible ferrichrome ABC transporter components | SA1977, 1978, and 1979 | None | Iron uptake | 77, 74, and 71 |
| Ferrichrome ABC transporter FhuD homolog | SA2079 | *fhuD* | Iron uptake | 84 |
| Possible iron transport proteins | SA2337 and 2369 | None | Iron uptake | 80, 70 |
| Ferrichrome ABC transporter FhuD homolog | SAV0812 | SaGIm* | Iron uptake | — |

Others

| ORFs | Gene name | Function | Similarity |
|------|-----------|----------|------------|
| SA0144-159 | *cap A-P* | Possible escape from immune system | 72–89 cap A-D, M,P |
| SA0126, 0127 | Unknown | | — |
| SA2459, 2460, 2461, 2462, and 2462 | *icaA, D, B, and C* | Cell-cell aggregation on infected tissues | — |
| SA0841, 1750-51, 2006 | Unknown | | — |
| SA0102 | Potential immune disorder in host | | — |
| SA1193 | *mprF (fmtC)* | Resistance to cationic antimicrobial peptides | 75 |
| SA0793-96 | *dltA, B, C, and D* | Resistance to cationic antimicrobial peptides | 89, 84, 99, and 84 |

Symbol and abbreviations: −, not determined; ORFs, open reading frames; ABC, ATP-binding cassette; and MHC, major histocompatibility complex.

*SaGIm is the genomic island name.

*Gene is widespread.

Blue indicates newly identified ORFs.

Red indicates counterpart in the *S. carnosus* TM300 genome.
infection. Compared to *S. aureus*, *S. carnosus* was also found to have a much decreased number of other potential virulence factors such as exoenzymes and adhesins.

This is only a preliminary study since the *S. epidermidis* and *S. carnosus* genomes were only screened for the presence of known *S. aureus* virulence genes. On the other hand, not many virulence genes are known in *S. epidermidis*. However, this first analysis may shed some light why *S. epidermidis* causes rather mild or chronic infections. In the future, we shall get a more complete picture if the genomes of other staphylococcal species are included in the comparative genome analysis.

### The Regulatory Network in Staphylococci

**Accessory Gene Regulation**

The initial step in an infectious disease is often adhesion to and colonization of host tissue surfaces. *Staphylococcus aureus* has been shown to bind to several host matrix proteins and plasma proteins, such as fibronectin (Flock et al., 1987; Froman et al., 1987; Jonsson et al., 1991), fibrinogen (Boden and Flock, 1989), collagen (Foster and Höök, 1998), elastin (Park et al., 1991), laminin (Vercellotti et al., 1985; Herrmann et al., 1988), prothrombin (Kawabata et al., 1985), thrombospondin (Herrmann et al., 1991), bone sialoprotein (Ryden et al., 1990; Yacoub et al., 1994), and vitronectin (Liang et al., 1995; Heilmann et al., 1997). For each of these binding functions, a corresponding surface-associated protein has been identified. The existence of an *S. aureus* extracellular matrix binding protein with broad specificity that is capable of binding several extracellular glycoproteins has also been reported (McGavin et al., 1993). The role of some of these proteins in the pathogenesis of staphylococcal infections has been shown in animal models (Foster and McDevitt, 1994; Moreillon et al., 1995; Flock et al., 1996; Palma et al., 1996).

The expression of many matrix-binding proteins is controlled by the agr (accessory gene regulator) system. Many of the genes coding for extracellular toxins, enzymes, and cell surface proteins in *S. aureus* are regulated by a 510-nucleotide RNA molecule, RNAIII. Transcription of genes encoding secreted toxins and enzymes (including *hla* [α-toxin], *saeB* [enterotoxin B], *tst* [toxic shock syndrome toxin 1], and *ssp* [serine protease]) is stimulated, while transcription of genes encoding cell surface proteins (like *spa* [protein A] and *fnb* [fimbriebinding proteins]), is repressed (Tegmark et al., 1998).

In *S. aureus*, transcription of at least 15 virulence genes, encoding extracellular toxins, enzymes, and cell surface proteins, is also regulated (Janzon and Arvidson, 1990; Novick et al., 1993). Production of toxins and enzymes is generally positively controlled, while that of cell surface proteins is negatively controlled (Janzon et al., 1989; Kornblum et al., 1990). In Table 21, agr-controlled genes are classified according to their degree of regulation (Kornblum et al., 1990). For example, the expression of α-hemolysin, serine protease, toxic shock syndrome toxin (TSST-1), and δ-lysin is enhanced in an agr+ and very low

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**Table 20. Presence of antibiotic resistance genes in *S. aureus* N315/Mu50 and *S. carnosus* TM300.**

| Resistance genes                  | ORF  | Gene name | Function    | Similarity (%) |
|-----------------------------------|------|-----------|-------------|----------------|
| Penicillin binding protein 29     | SA0038| mecA      | Pc resistance | —              |
| β-Lactamase                       | SAP010| blaZ      | Pc resistance | —              |
| rRNA adenine N-6-methyltransferase| SA0048| ermA      | Em resistance | —              |
| rRNA adenine N-6-methyltransferase| SA0766| ermA      | Em resistance | —              |
| rRNA adenine N-6-methyltransferase| SA1480| ermA      | Em resistance | —              |
| rRNA adenine N-6-methyltransferase| SA1951| ermA      | Em resistance | —              |
| rRNA adenine N-6-methyltransferase| SA2348| ermA      | Em resistance | —              |
| O-nucleotidyltransferase          | SA0049| ant       | Ags resistance | —              |
| O-nucleotidyltransferase          | SA0765| ant       | Ags resistance | —              |
| O-nucleotidyltransferase          | SA1481| ant       | Ags resistance | —              |
| O-nucleotidyltransferase          | SA1952| ant       | Ags resistance | —              |
| O-nucleotidyltransferase          | SA2385| ant       | Ags resistance | —              |
| Bifunctional AAC/APH protein      | SAVP026| aacA      | Ags resistance | —              |
| Tetracycline resistance protein   | SA0387| tetM      | Tc resistance | —              |
| Bleomycin resistance protein      | SA0032| bleO      | Ble resistance | —              |

Symbol and abbreviations: —, not determined; ORF, open reading frame; Pc, penicillin; Em, erythromycin; Ags, aminoglycoside; Tc, tetracycline; Ble, bleomycin; and AAC/APH, 6′-aminoglycoside-N-acetylttransferase and 2′-aminoglycoside phosphotransferase.

*Two copies.

*Three copies.
in an agr\(^-\) background, while the expression of the cell wall-bound proteins such as Protein A, coagulase, and fibronectin BP is downregulated. Synthesis of RNAIII is induced when the concentration of an autocrine octapeptide in the environment has reached a certain level (Ji et al., 1995; Ji et al., 1997). Generally, this happens during the late exponential phase of growth in laboratory cultures, which means that cell surface proteins are produced during the early exponential phase, while secreted toxins and enzymes are produced mainly during the post-exponential phase of growth (Björkling and Arvidson, 1980; Vandenesch et al., 1993; Lebeau et al., 1994).

The agr locus contains two divergent transcription units, RNAII and RNAIII, driven by the promoters P2 and P3, both of which are active only from the mid-exponential phase of growth (Novick et al., 1995). RNAII contains four genes: \(agrA\), \(agrB\), \(agrC\) and \(agrD\). The \(agrC\) and \(agrA\) genes code for the components of a classical two-component signal transduction system, where AgrC is the membrane-bound signal receptor and AgrA is the response regulator, which is required for transcription of the RNAIII molecule and the agr operon itself (Novick et al., 1993; Novick et al., 1995). The autoinducing peptide (AIP) is encoded in and derived from the AgrD prepeptide that is in an unknown way post-translationally processed, modified and secreted. Very likely AgrB (Ji et al., 1995; Ji et al., 1997), an integral membrane protein, is involved besides additional factors (Saenz et al., 2000). The resultant mature AIP is the ligand that binds to and activates the phosphorylation of AgrC (Lina et al., 1998), which in turn is thought to phosphorylate AgrA, and phosphorylated AgrA found to induce RNAII and RNAIII synthesis (Ji et al., 1995). RNAIII causes an upregulation of secreted virulence factors as well as the downregulation of surface proteins. The gene organization of the agr system is shown in Fig. 16.

| Class | Exoprotein | Gene   | agr\(^+\) | agr\(^-\) |
|-------|------------|--------|----------|----------|
| I     | \(\alpha\)-hemolysine | \(hla\) | high     | undetectable |
|       | serine protease      | \(spr\) (V8) | high     | undetectable |
|       | TSS-1                | \(tst\) | high     | undetectable |
|       | \(\delta\)-hemolysin | \(hld\) | high     | undetectable |
| II    | nuclease             | \(nuc\) | high     | low-moderate |
|       | \(\beta\)-hemolysin  | \(hbb\) | high     | low-moderate |
|       | Enterotoxin B        | \(entB\) | high     | low-moderate |
| III   | Protein A            | \(spa\) | low      | high     |
|       | Coagulase            | \(coa\) | low      | high     |
|       | FibronectinBP        | \(fba\) | low      | high     |
| IV    | \(\beta\)-Lactamase   | \(bla\) | no effect |         |
|       | Enterotoxin A        | \(entA\) | no effect |         |

Fig. 16. Accessory gene regulatory (agr) genes and regulation cascade. From promoter region (P2), the RNA-II is transcribed encoding the genes \(agrB, agrD, agrC\) and \(agrA\). The thiolactone peptide acts as an autorepressor that is sensed by the two component proteins AgrC and AgrA; the latter in its phosphorylated state activates transcription from P2 and P3. RNA-III controls gene expression of secreted and cell-bound proteins in an essentially unknown manner. RNA-III transcription is also positively controlled by the staphylococcal accessory regulator (SarA).

**Quorum Sensing**

The synthesis of RNAIII is regulated by a quorum sensing mechanism. The AIP molecules accumulate in the culture supernatant, and when a certain threshold concentration is reached, they interact with AgrC and induce as a consequence both RNAII and RNAIII transcription. Since transcription of P2 requires P2 operon products, the P2 operon is autocatalytic, and is thus suited for rapid production of secreted proteins at a time when overall growth is coming to a halt.

RNA III is the actual effector of the agr response, and incidentally encodes the agr-regulated peptide \(\delta\)-lysin (Janzon et al., 1989), which is a 26-amino acid polypeptide that can form pores in membranes and lyse erythrocytes.
(Freer and Birkbeck, 1982) but is not required for the regulation of target genes by RNAIII. How RNAIII interacts with target gene promoters is unknown.

Distribution of the Agr Locus

The agr locus, organized in the same way as that of S. aureus, has been demonstrated in the coagulase-negative S. lugdunensis (Vandenesch et al., 1993). However, RNAIII from S. lugdunensis does not code for ß-lysin, and its role in gene regulation is not known. The agr genes are also present in other coagulase-negative staphylococci such as S. epidermidis, S. simulans and S. warneri where RNAIII and ß-lysin homologues have been identified (Otto et al., 1998; Tegmark et al., 1998). In all RNAIII molecules, the first 50 and last 150 nucleotides were highly conserved, suggesting that these regions are important for the regulatory function.

Ag-specific Peptide Pheromones are Thiolactones

The agr-specific peptide pheromones are referred to in the literature as AIP. However, the latter designation, does not consider the findings that these peptide pheromones not only induce the native agr system but also act at the same time as inhibitors of foreign agr systems. Therefore, the less restrictive term “peptide pheromone” is preferred.

The first hint that a peptide acts as an autoinducer of the agr system in S. aureus comes from the group of R. Novick. It turned out that the staphylococcal peptide pheromone sequences that are encoded in agrD gene vary from species to species. Even within S. aureus, four subtypes were identified (Ji et al., 1997). The first structure of a pheromone peptide and its biological activity was described in 1998 (Otto et al., 1998). The peptide sequence, DSVCASYF, was derived from AgrD of S. epidermidis. This synthetic octapeptide revealed only autoinducing activity when it contained the thiolactone ring between the central cysteine and the C-terminal carboxyl group (DSVc[CASYF]). The structure of the peptide pheromone of S. epidermidis is shown in Fig. 17. Unmodified peptides showed no activity (Otto et al., 1998). The receptor for the peptide pheromones, AgrC, must be very sensitive since the thiolactones are active at nanomolar concentrations. The N-terminus of the peptide pheromones is crucial for biological activity and specificity (Otto et al., 1998).

Interestingly, the S. epidermidis specific peptide pheromone is a potent inhibitor of the S. aureus agr system (Otto et al., 1999). However, while the correct N-terminus and the thiolactone structure were absolute prerequisites for an agr-activating effect in S. epidermidis, inhibition of the S. aureus agr system showed a lesser structural stringency. Another important finding was that the corresponding lactone- and lactam-containing peptides DSVc[SASYF] and DSVc[DprASYF] inhibit the S. aureus agr system, but have lost autoinducing activity in its native S. epidermidis agr system (Otto et al., 1999). The S. epidermidis thiolactone inhibits the agr subgroups 1–3 but not 4 (Otto et al., 2001). An explanation for this could be that subgroup 4 thiolactone is very similar to that of S. epidermidis. A series of autoinducing peptide analogues (including the L-alanine and D-amino acid scanned peptides) were synthesized to determine the functionally critical residues within the S. aureus group I. It was found that 1) the group I autoinducing peptide (YSTc[CDFIM]) is inactivated in culture supernatants by the formation of the corresponding methionyl sulfoxide, and 2) that the lactam analogue retains the capacity to activate agr, suggesting that the thiolactone structure, which would allow covalent modification of the AgrC receptor, is not a necessary prerequisite for agr activation. Replacement of the endocyclic amino acid residue (D-aspartate) with alanine converted the group I thiolactone from an activator to a potent inhibitor. The addition of exogenous agr inhibitors to S. aureus decreased the production of TSST-1 and enterotoxin C3, confirming the potential of quorum-sensing blockade as a therapeutic strategy (Dowell et al., 2001).

It is very likely that in nature, there is a fierce competition between S. aureus and S. epidermidis and it looks like quorum-sensing cross talk generally favors survival of S. epidermidis, which might explain the predominance of S. epidermidis on the skin and in infections on indwelling medical devices (Götz, 2002).

Agr and Pathogenicity

In agr mutants, decrease in pathogenicity is very likely due to decreased synthesis of extracellular toxins and enzymes, such as α-, β-, and
δ-hemolysins, leucocidin, lipase, hyaluronate lyase, and proteases. In a murine arthritis model, the virulence was investigated of \textit{S. aureus} 8325-4 and two \textit{agr/hld} mutants derived from it (Abdelnour et al., 1993). Sixty percent of the mice injected with the wildtype strain developed arthritis, whereas \textit{agrA} and \textit{hld} mutants displayed joint involvement in only 10 and 30%, respectively. The question is also whether \textit{agr} inhibiting thiolactone peptides can be therapeutically used. First tests showed that \textit{agr} inhibitors exhibited biological activity in vivo in a mouse protection test (Mayville et al., 1999).

**Staphylococcal Accessory Regulator**

In \textit{S. aureus}, the production of virulence factors such as cell wall adhesins and exotoxins during the growth cycle has been shown to be under the control of at least two regulator genes, \textit{agr} and staphylococcal accessory regulator (\textit{sar}; Manna et al., 1998). The global \textit{agr} regulatory system is itself controlled by another regulator, namely the \textit{sis} regulon system. This regulation system was first identified by Ambrose Cheung and his colleagues (Cheung et al., 1992). The \textit{sar} locus consists of the 372-bp \textit{sarA} preceded by a triple promoter region interspersed with two smaller open reading frames (ORFs) ORF3 and ORF4 (Bayer et al., 1996). The triple promoter system yields three overlapping \textit{sar} transcripts, \textit{sarA}, \textit{sarC} and \textit{sarB} of 0.56, 0.8 and 1.2 kb, respectively (Fig. 18). Expression studies with a \textit{sigB} mutant revealed that the P3 promoter is \textit{SigB} dependent (Manna et al., 1998). The protein sequence of the 14-kDa SarA shares sequence similarity with VirF of \textit{Shigella flexneri} (Cheung and Projan, 1994).

Mutations in \textit{sarA} resulted in a decreased expression of several extracellular (especially \textit{α}-hemolysin) and cell wall proteins. This can be explained by the fact that transcription of RNAII and RNAIII of the agr system is also decreased. The \textit{sarA} mutants can be complemented in part by plasmids expressing SarA, however, a full complementation was only achieved if \textit{sarB} or \textit{sarC} is expressed. These data suggest that ORF3, and to a lesser degree ORF4, may affect \textit{agr} expression by modulating SarA protein expression. SarA binds to the P2 promoter region of \textit{agr}, thus leading to transcription of RNAII and, subsequently, RNAIII, the two major transcripts encoded within the \textit{agr} locus (Chien et al., 1998). The reduction in SarA expression correlated with a lower level of \textit{agr} activation in the corresponding \textit{sar} mutant clone.

However, in addition to controlling target gene expression via \textit{agr} (e.g., \textit{α}-hemolysin), the \textit{sar} locus can also regulate target gene transcription via \textit{agr}-independent mechanisms. In particular, it was found that SarA binds to conserved sequences homologous to the SarA-binding site on the \textit{agr} promoter, upstream of the –35 promoter boxes of several target genes including \textit{hla} (\textit{α}-hemolysin gene), \textit{spa} (protein A gene), \textit{fnb} (fibronectin-binding protein genes), and \textit{sec} (enterotoxin C gene; Chien et al., 1999). Deletion of the SarA recognition motif in the promoter regions of \textit{agr} and \textit{hla} in shuttle plasmids rendered the transcription of these genes undetectable in \textit{agr} and \textit{hla} mutants, respectively. Likewise, the transcription activity of \textit{spa} (a gene normally repressed by \textit{sar}) became derepressed in a wildtype strain containing a shuttle plasmid in which the SarA recognition site had been deleted from the \textit{spa} promoter region. It is assumed that SarA is a regulatory protein that binds to its consensus recognition motif to activate (e.g., \textit{hla}) or repress (e.g., \textit{spa}) the transcription of \textit{sar} target genes, thus accounting for both \textit{agr}-dependent and \textit{agr}-independent mode of regulation (Chien et al., 1999).

With a number of selected promoter regions, the one under control of SarA was investigated. Of the seven \textit{σ(A)}-dependent promoters that were tested, SarA repressed transcription from \textit{agrP2}, \textit{agrP3}, \textit{cna}, \textit{sarP1}, and \textit{sea} promoters and did not affect \textit{sec} and \textit{znt} promoters. Furthermore, SarA had no effect on transcription from the \textit{σ(B)}-dependent \textit{sarP3} promoter. In vitro experimental data presented in this report suggest that SarA expression is autoregulated (Chakrabarti and Misra, 2000). SarA binds to conserved DNA motifs immediately upstream of both positively and negatively regulated promoters.

SarA interacts with a series of heptad repeats (AGTAAAG) within the \textit{agr} promoter. Subsequent DNA-binding studies revealed that SarA binds readily to multiple AT-rich sequences of variable lengths. Crystal structure analysis of SarA and a SarA-DNA complex brought some insight to bear on the conformational changes

![Fig. 18. Staphylococcal accessory regulator (sar) locus. The sar locus consists of the 372-bp sarA preceded by a triple promoter region (P1-P3) interspersed with two smaller open reading frames (ORF3 and ORF4; Bayer et al., 1996). The triple promoter system yields three overlapping sar transcripts, sarA, sarC and sarB of 0.56, 0.8 and 1.2 kb, respectively.](image-url)
resulting from SarA encasement of DNA. SarA recognizes an AT-rich site in which the DNA is highly overwound and adopts a D-DNA-like conformation by indirect readout (Schumacher et al., 2001).

SarA Protein Family

The staphylococcal genome sequences revealed a number of SarA homologs. In search of additional regulators that could explain the differential effects of RNAIII and SarA, four differently regulated genes (hla, α-toxin; hld, RNAIII; spa, protein A; and ssp, serine protease) were analyzed for binding of potential regulatory proteins to the corresponding promoter DNA fragments linked to magnetic beads. One protein (29 kDa), with affinity for all four promoters, showed a high degree of similarity to SarA and was named “SarH1” (Sar homolog 1). Expression of sarH1 was strongly repressed by sarA and agr. Further analysis revealed that sarH1 has a strong repressive effect on hla and an activating effect on spa transcription (Tegmark et al., 2000). Furthermore, the production of several other exoproteins was affected by sarH1.

The level of SarA is partially controlled by the differential activation of sar promoters. With a DNA-specific column containing a sar P2 promoter fragment, a protein was purified which is encoded by sarR. SarR is a 13.6-kDa protein with homology to SarA. SarR binds to sar P1, P2, and P3 promoter fragments and a sarR mutant expressed a higher level of P1 transcript than the parent. As the P1 transcript is the predominant sar transcript, it was proposed that SarR is a sarA repressor protein that binds to the sar promoters to downregulate P1 transcription and, consequently, SarA expression (Manna and Cheung, 2001).

The genome sequence revealed another SarA homolog, the 250-amino-acid (aa) SarS. Its gene is upstream of the spa gene. The expression of sarS was almost undetectable in parental S. aureus strain but was highly expressed in agr and sarA mutants, strains normally expressing a high level of protein A. Interestingly, protein A expression was decreased in a sarS mutant. The enhancement in spa expression in an agr mutant returned to a near-parental level in the agr-sarS double mutant, but not in the sarA-sarS double mutant. All the data indicated that agr probably mediates spa repression by suppressing the transcription of sarS, an activator of spa expression. However, the pathway by which the sarA downregulates spa expression is sarS independent (Cheung et al., 2001).

Another SarA homolog is the 118-aa long and basic SarT. Expression of sarT is repressed by sarA and agr. In a sarT mutant, the RNAIII level was notably increased particularly in the postexponential phase. SarT repressed the expression of hla (α-toxin) similar to the repression in agr and sarA mutants. This finding suggests that sarA, contrary to the regulatory action of agr, induced α-hemolysin production by repressing sarT, a repressor of hla transcription (Schmidt et al., 2001).

Many of the SarA protein family members are either small basic proteins (<153 residues) or two-domain proteins in which a single domain has sequence similarity with one of the small basic proteins. Because of its structure and unique mode of DNA binding, SarR, and possibly other SarA family members, may belong to a new functional class of the winged-helix family, accommodating a long stretch of DNA with bending points. On the basis of sequence homology, it is hypothesized that the SarA protein family may entail homologous structures with similar DNA-binding motifs but divergent activation domains. An understanding of how these regulators interact with each other in vivo and how they sense environmental signals to control virulence gene expression (e.g., α-hemolysin) will provide more insight in the complex regulatory network (Cheung and Zhang, 2002).

Exoprotein Gene Regulator

In addition to agr and sar, another exoprotein gene regulator (sae) is involved in the expression of extracellular and cell surface proteins. In sae mutants, the production of α- and β-hemolysins and coagulase are drastically diminished and to a lesser extent also protein A. The sae locus regulates these exoprotein genes at transcriptional level (Giraudo et al., 1997). The sae mutation does not affect the expression of agr or sar. A sae-agr double mutant expressed reduced or null levels of α-, β-, and δ-hemolysins, coagulase, and high levels of protein A. The corresponding genes are not transcribed, while spa is transcribed at high levels. It is assumed that sae and agr interact in a complex way in the control of the expression of the genes of several exoproteins. The sae locus consists of two genes, designated “saeR” and “saeS,” encoding a typical response regulator and histidine protein kinase, respectively (Giraudo et al., 1999).

In a guinea pig model of device-related infection, the impact of agr, sarA and sae mutants on the induction of hla (α-toxin gene) transcription was studied (Goerke et al., 2001). Staphylococcus aureus strains RN6390 and Newman expressed considerably smaller amounts of RNAIII in the guinea pig than during in vitro growth. Highest RNAIII and hla expression was detected in both strains early in infection, decreased during the course of infection that suggests that it was negatively correlated with
bacterial densities. The *agr* and *sarA* mutants of strains Newman and RN6390 did not affect *hla* expression in vivo, while in *sae* mutants, *hla* is severely downregulated in vitro as well as in vivo. This study suggests that *S. aureus* seems to be provided with regulatory circuits different from those characterized in vitro to ensure α-toxin synthesis during infections (Goerke et al., 2001).

**Repressor of Toxins**

Recently a gene called “repressor of toxins” (*rot*) was identified in *S. aureus* RN6390 that shows homology with *agrA* and *sarA* (McNamara et al., 2000). In a *rot-agr* double mutant the expression of protease and α-toxin is restored. This phenotype suggests that Rot acts as a transcriptional repressor of *hla*. Whether other genes are controlled by Rot is unknown.

**The arlS-arlR Two-component System**

In a *S. aureus* 8325 derivative, another two-component system, *arlS-arlR*, was identified which is involved in regulation of exoproteins (Fournier and Hooper, 2000a). ArlS is the sensor protein while ArlR represents the response regulator. An *arlS* mutant showed pleiotropic effects. It formed a biofilm on a polystyrene surface unlike the parent strain and the complemented mutant strain. Biofilm formation was associated with increased primary adherence to polystyrene, whereas cellular adhesion was only slightly decreased. In addition, the *arlS* mutant exhibited increased autoolysis and altered peptidoglycan hydrolase activity. Another effect of the *arlS* mutant is that the activity of multidrug resistance efflux pump, NorA, is increased (Fournier et al., 2000b). While in the *arlS* or *arlR* mutants, the extracellular proteolytic activity, including serine protease activity, is dramatically decreased (Fournier and Hooper, 2000a), and transcription and production of other secreted proteins such as α- and β-hemolysins, lipase, coagulase, or the cell-wall bound protein A are increased (Fournier et al., 2001). The *arl* operon decreases the production of virulence factors by transcriptional downregulation of the corresponding genes. Since the *arl* mutation did not change *spa* expression in an *agr* or *sarA* mutant, it is suggested that *arl* acts indirectly on virulence gene expression through *agr* and *sar*. This is supported by the finding that RNAII and RNAIII are decreased by ArlS-ArlR, while *sarA* expression is increased.

**Staphylococcal Virulence Regulator**

The staphylococcal virulence regulator gene *svrA* was originally identified in *S. aureus* by signature-tagged mutagenesis as necessary for virulence. SvrA is a membrane-associated protein, having two regions with six membrane-spanning domains, separated by an extended hydrophilic loop (Garvis et al., 2002). The *svrA* mutant expressed greatly reduced amounts of α-, β- and δ-toxins and an increased amount of protein A; the regulation occurred at the transcriptional level. As the *agr*-specific RNAII and RNAIII were absent in the *svrA* mutant, it is assumed that SvrA is, like many other regulators, required for expression of *agr*.

**The SrrA-SrrB Two-component System**

It was found that the expression of toxic shock syndrome toxin 1 (TSST-1) by *S. aureus* in liquid culture consumes oxygen. In the course of studies examining the mechanism by which oxygen might regulate toxin production, the *srrAB* (staphylococcal respiratory response) genes were identified. The genes are homologous to the *B. subtilis* resDE genes. ResD-ResE represents a global regulator of aerobic and anaerobic respiratory metabolism in *B. subtilis*. The two-component regulatory system SrrA-SrrB very likely acts in anaerobic repression of staphylococcal virulence factors (Yarwood et al., 2001). In *srrB* mutants, RNAIII synthesis was upregulated while exotoxin TSST-1 synthesis was downregulated under microaerobic conditions and, to a lesser extent, under aerobic conditions as well. At the same time, protein A production was upregulated in microaerobic conditions and decreased in aerobic conditions. Overexpression of *srrAB* resulted in nearly complete repression of TSST-1 production in both microaerobic and aerobic conditions. It is assumed that SrrA-SrrB acts in the global regulation of staphylococcal virulence factors, and may repress virulence factors under low-oxygen conditions. Furthermore, *srrAB* may provide a mechanistic link between respiratory metabolism, environmental signals, and regulation of virulence factors in *S. aureus* (Yarwood et al., 2001). This regulation is mediated in part by *agr*.

**The Alternative Sigma Factor: Sigma B**

In *S. aureus*, homologous genes to the *sigB* operon of the stationary-phase σ factor SigB (sigB) of *Bacillus subtilis* were identified (Kullik and Giachino, 1997). The *sigB* region contains a total of six ORFs of which orf2, orf3, orf4 and orf5 show 64, 67, 71 and 77% similarity to the *B. subtilis* proteins RsbU, RsbV, RsbW and SigB, respectively, with SigB representing the σ factor and the Rsb proteins representing regulators of SigB. The organization of the *sigB* operon is shown in Fig. 19.
Very likely we have in *S. aureus* a similar sigB regulation cascade as described for *B. subtilis* (for review, see Mittenhuber, 2002). In *B. subtilis*, SigB is already present in unstressed cells (e.g., in exponential growth phase), but it is bound and sequestered by the anti-σ factor RsbW and therefore unable to interact with the core-enzyme of RNA polymerase (Hecker and Volker, 1998; Scott et al., 2000). A third protein, the anti-anti-σ factor RsbV, which can also bind to RsbW, accomplishes release of SigB from RsbW. The phosphorylation status of RsbV is critical for its binding to RsbW. In its unmodified form, RsbV forms a complex with RsbW, whereas P-RsbV is unable to bind to RsbW that can then form a complex with SigB. This novel regulatory principle was called “partner switching” (Alper et al., 1994). Antagonistic activities of a kinase and two phosphatases control the phosphorylation status of RsbV. In addition, RsbW is also an RsbV kinase, whereas RsbU (phosphatase) dephosphorylates P-RsbV. Depending on the nature of the growth-restricting factor, RsbU is activated after imposition of environmental (physical) stress.

However, in *B. subtilis*, other genes are involved in stress regulation (e.g., *rsbS, rsbR, rsbT* and *rsbX*) that are not identified in *S. aureus* so far. Therefore, one has to be cautious in the unexamined functional adaptation of the homologous Rsb proteins.

Deletion of *sigB* in *S. aureus* revealed its function as a global regulator of virulence genes (Kullik et al., 1998a). The *sigB* mutants showed reduced pigmentation, accelerated sedimentation, and increased sensitivity to hydrogen peroxide during the stationary growth phase. Furthermore, the cytoplasmic alkaline shock protein 23 and pigmentation were undetectable, while some secreted enzymes such as thermonuclease and lipase were increased in the mutant. The *S. aureus* *rsbW* encodes an anti-σ factor of SigB and acts as a negative posttranslational regulator (Miyazaki et al., 1999). In an in vitro transcription runoff assay, RsbW prevented SigB-directed transcription from the sarA-P3 promoter, a known SigB-dependent promoter.

To find out the influence of a functional *sigB* on the global regulators *sar* and *agr*, studies were carried out in the *sigB*-positive MSSA1112 and Newman strains carrying the wildtype *rsbU* allele (Bischoff et al., 2001). The SigB concentration reached a maximum in the late exponential phase and declined towards the stationary phase when bacteria were grown in Luria-Bertani medium. In *sarP1-3* reporter fusion studies, a strong SigB and growth phase-dependent increase in *sar* expression that was totally absent in either *rsbU* or *rsbU-sigB* deletion mutants was revealed. In contrast, expression of agr RNAIII is increased in the *sigB* mutant. Thus, SigB increases *sar* expression while simultaneously reducing the RNAIII level in a growth phase-dependent manner.

Interestingly, most *sigB* deletion phenotypes were only seen in *S. aureus* COL and Newman and not in 8325, which was found to contain an 11-bp deletion in the regulator gene *rsbU* and behaves phenotypically like a *sigB*-defective mutant. Since hydrogen peroxide (*H₂O₂*) represents an important stress factor for *S. aureus* during infection, it is postulated that *sigB* is important to survive high concentrations of peroxide especially during late stationary phase. The genes that are controlled by SigB (such as *asp23*) contain a SigB-specific promoter consensus sequence.

**Critical Remarks**

There is no doubt that the accessory gene regulator (agr) and the staphylococcal accessory regulator (sar) are central regulatory elements that control the production of virulence factors. Most of the functions of these global regulators have been defined using *S. aureus* RN6390, which is a representative of the laboratory strain 8325-4. However, RN6390 has a mutation in *rsbU* that results in a phenotype resembling that of a *sigB* mutant (Kullik et al., 1998b). For that reason, it remains unclear whether the regulatory characteristics described for RN6390 are representative for clinical isolates. To address this question, mutations were generated in the *sarA* and *agr* loci of three laboratory strains (RN6390, Newman, and S6C) and four clinical isolates (UAMS-1, UAMS-601, DB, and SC-1) and tested for collagen binding (*cna-expression*). The *sarA* mutants of strains UAMS-1 and UAMS-601 showed an increased capacity to bind collagen, while mutation of *agr* had little impact. Northern blot analysis confirmed that the increase in collagen binding was due to increased *cna* transcription. Without exception, an increased production
of proteases and a decreased capacity to bind fibronectin was revealed in sarA mutants. Mutation of agr had the opposite effect. Although sarA mutants had slightly reduced fnbA transcription, changes in the ability to bind fibronectin appeared to be more directly correlated with changes in protease activity. Lipase production was reduced in both sarA and agr mutants.

In contrast to the SigB-positive strains, mutation of sarA in RN6390 (affected in rsbU) resulted in a reduced hemolytic activity. The levels of the sarC transcript also seemed to be reduced in RN6390, but there was no difference in the overall pattern of sarA transcription or the production of SarA. Taken together, these results suggest that studies defining the regulatory roles of sarA and agr by using RN6390 are not always representative of the events that occur in clinical isolates of *S. aureus* (Blevins et al., 2002).

**Ecology**

Members of the genus *Staphylococcus* are widespread in nature and occupy a variety of niches. As a result of their ubiquity and adaptability, staphylococci are a major group of bacteria inhabiting the skin, skin glands, and mucous membranes of humans, other mammals, and birds. A variety of habitats present in the human cutaneous ecosystem can be distinguished by differences in the density and structure of the microbial communities inhabiting them, as well as by their anatomical and physiological properties. For example, skin regions supplied with large numbers of pilosebaceous units, sweat glands, and mucous membranes surrounding openings to the body surface contain the largest populations of staphylococci. In addition, staphylococcal communities may be found living in the follicular canals, the openings to sweat glands, the capacious lumen of sebaceous follicles, and on the surface of and beneath desquamating epithelial scales (Noble and Somerville, 1974; Noble and Pitcher, 1978).

Staphylococci may be found on the skin as residents or transients; as a result, one must use stringent criteria based on temporal studies and population size to estimate residency status and host range (Price, 1938; Noble and Somerville, 1974; Kloos and Musselwhite, 1975a). Resident bacteria are indigenous to the host, maintain relatively stable populations, and increase in numbers mainly by multiplication of those already present. Transient bacteria are derived from exogenous sources, found primarily on exposed skin, and may be easily washed away. Cross-contamination of staphylococci can occur readily where different host species come in contact with one another, but where host specificity is high, the transient organisms will usually be eliminated within several hours or days, unless the normal defense barriers are compromised. Ideally, determination of natural host range should be made with host species that are relatively isolated in nature. Most of the ecological studies reported fall short of this ideal situation, but a few have indicated some clear patterns of host and niche preferences for certain *Staphylococcus* species.

*Staphylococcus epidermidis* (Schleifer and Kloos, 1975b) is the most prevalent and persistent *Staphylococcus* species on human skin. It is found over much of the body surface and produces the largest populations where moisture content and nutrition are high, such as in the anterior nares, axillae, inguinal and perineal area, and toe webs. This species may be found occasionally on other hosts, such as domestic animals, but it is presumably transferred there from human sources. *Staphylococcus hominis* (Kloos and Schleifer, 1975b) is also prevalent on human skin. Its population size is usually second or equal to *S. epidermidis* on skin sites where apocrine glands are numerous (e.g., in the axillae and inguinal and perineal areas). It can also colonize the drier regions of skin (e.g., on the extremities) more successfully than other species. *Staphylococcus haemolyticus* (Schleifer and Kloos, 1975c) shares many of the habitats of *S. hominis*, but it is usually found in smaller populations. Some individuals may carry unusually large populations of *S. haemolyticus*. A different subspecies of *S. haemolyticus* found living on nonhuman primate skin (e.g., Pan, Macaca, Cercocebus, Erythrocebus, Microcebus and Lemur skin) can be distinguished from the human-adapted subspecies on the basis of DNA-DNA hybridization (Kloos and Wolfshohl, 1979). Since it is difficult to distinguish colonies of the nonhuman primate *S. haemolyticus* subspecies from a sibling species, provisionally designated “*S. simians,*” found also on nonhuman primate skin, adequate enumeration of this subspecies is not yet possible. *Staphylococcus warneri* (Kloos and Schleifer, 1975b) is found usually in small numbers on human skin, though a few individuals may carry unusually large populations. *Staphylococcus warneri* is a major species on nonhuman primates, especially on the more advanced Cercopithecoida and Pongidae. Occasionally, small transient populations of *S. haemolyticus* or *S. warneri* may be isolated from domestic animals. *Staphylococcus capitis* (Kloos and Schleifer, 1975b) produces large populations on the human scalp following puberty. It is also found on other regions of the adult head, e.g., forehead, face, eyebrows, and external auditory
meatus in moderate-sized to large populations. The largest populations are found in areas where sebaceous glands are numerous and well developed. *Staphylococcus capitis* subsp. *ureolyticus* is present on regions of the head in rather small populations and, like *S. capitis*, may be found only occasionally on other body sites (Bannerman and Kloos, 1991). This subspecies has been isolated from both human and nonhuman primate skin (e.g., Pan skin).

*Staphylococcus caprae*, originally isolated from the skin of domestic goats or in their milk (Devriese et al., 1983; Poutrel, 1984), has been isolated from human clinical specimens (Vandenesch et al., 1995; Kawamura et al., 1998). *Staphylococcus hominis* subsp. *novobiosepticus*, *S. lugdunensis*, *S. pasteuri*, *S. schleiferi* are other clinically significant species isolated from human specimens (Freney et al., 1988; Chesneau et al., 1993; Kloos et al., 1998b). Their original niche preference and prevalence is undetermined.

*Staphylococcus auricularis* is one of the major species found living in the adult human, external auditory meatus and demonstrates a strong preference for this niche (Kloos and Schleifer, 1983a). A different subspecies of *S. auricularis* is found in the ear and specialized scent (or marking) glands of nonhuman primates (e.g., *Pan, Pongo, Cercopithecus, Lemur, Galago* and *Microcebus*; Kloos, 1985; Kloos and Schleifer, 1986).

*Staphylococcus aureus* is a major species of primates, though specific ecovars or biotypes can be found occasionally living on different domestic animals or birds (Meyer, 1967; Kloos, 1980). This species is found infrequently on nonprimate wild animals. In humans, *S. aureus* has a niche preference for the anterior nares, especially in the adult. Here it can exist as a resident or as a transient member of the normal flora. *Staphylococcus aureus* selectively adheres to nasal epithelial (mucosal) cells (Aly et al., 1981). Nasal carrier rates range from less than 10% to more than 40% in normal adult human populations residing outside of the hospital (Noble and Somerville, 1974). The nasal adherence of *S. aureus* is significantly greater for carriers of this species than for noncarriers. *Staphylococcus aureus* subsp. *anaerobius* is found living on sheep (De la Fuente et al., 1985).

The host range of *Staphylococcus saprophyticus* and similar species varies from humans to lower mammals and birds (Kloos, 1980; Devriese, 1986). As a group, these staphylococci are most prevalent on lower primates and mammals. Those species found most frequently on primates include *S. saprophyticus*, *S. cohnii* and *S. xylosus*. *Staphylococcus saprophyticus* is found usually in small, transient populations on the skin of humans or other primates. This species possesses surface properties that allow it to adhere readily to urogenital cells (Colleen et al., 1979). It may also be isolated from lower mammals and environmental sources. *Staphylococcus saprophyticus* subsp. *bovis* is found in the anterior nares of cows (Hálek et al., 1996). *Staphylococcus cohnii* (Schleifer and Kloos, 1975c) is found as a temporary resident or transient on human skin, and *S. cohnii* subsp. *urealyticus* (Kloos and Wollshohl, 1991) is sometimes found on human skin, but it is often one of the major species and subspecies of nonhuman primates, especially the lower primates. The largest populations of subspecies are found living on the Tupaiidae, Prosimii, and Ceboidea (Kloos and Wollshohl, 1983b). A third subspecies is also found on the Ceboidea. *Staphylococcus xylosus* (Schleifer and Kloos, 1975c) is often found as a transient on the skin of lower primates and other mammals, and occasionally on birds (Kloos et al., 1976b; Akatov et al., 1985; Devriese et al., 1985). The related species *S. kloosii* has been found living on a variety of lower mammals including wild marsupials, rodents and carnivores, and less frequently on domestic animals (Schleifer et al., 1984; Kloos, 1980). *Staphylococcus arlettae* has been isolated from poultry and goats, *S. equorum* from horses, and *S. gallinarum* from poultry (Devriese et al., 1983; Schleifer et al., 1984).

*Staphylococcus intermedius* is a major species of the domestic dog (Hálek and Marsalek, 1976b; Krogh and Kristensen, 1976). This species can be found in relatively large populations on canine skin and can on occasion be transferred to the skin of human handlers (Kloos et al., 1976b). *Staphylococcus intermedius* appears to be also indigenous to a variety of other carnivores, including the mink (Mustela; Hálek et al., 1972; Oeding et al., 1973; Hálek, 1976a), fox (Vulpes; Hálek, 1976a), and raccoon (Procyon; Kloos et al., 1976b). It has also been isolated from horses and pigeons (Hálek and Marsalek, 1971; Hálek, 1976a). *Staphylococcus felis* is one of the major species of the domestic cat (Igimi et al., 1989). *Staphylococcus schleiferi* subsp. *coagulans* is a coagulase-positive species, which has been isolated from the external auditory meatus of dogs with ear infections (Igimi et al., 1990). Other coagulase-positive species isolated from animals include *S. delphini* (Varaldo et al., 1988) and *S. lutrae* (Foster et al., 1997). *Staphylococcus sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* have been isolated from a variety of lower mammals and domestic animals (Kloos et al., 1976a; Kloos et al., 1997). In addition, the *S. sciuri* subspecies may be isolated from human clinical specimens (Marsou et al., 1983).
Opportunistic Pathogens

The coagulase-positive species *S. aureus*, *S. intermedius*, *S. delphini*, *S. schleiferi* subsp. *coagulans* and the coagulase-variable species *S. hyicus* are regarded as potentially serious pathogens. *Staphylococcus aureus*, since its early discovery as an opportunistic pathogen, continues to be a major cause of mortality and is responsible for a variety of infections. In the late 1950s and early 1960s, *S. aureus* caused considerable morbidity and mortality as a nosocomial pathogen. Among the major human infections caused by this species are furuncles, carbuncles, impetigo, toxic epidermal necrolysis (scalded skin syndrome), pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, cerebritis, meningitis, bacteremia, toxic shock syndrome, and abscesses of the muscle, skin, urogenital tract, central nervous system, and various intraabdominal organs. In addition, staphylococcal enterotoxin is involved in food poisoning. Methicillin-resistant *S. aureus* (MRSA) strains have emerged in the 1980s as a major clinical and epidemiological problem in hospitals. These strains are beginning to spread out of the hospitals and into communities. The origin and future significance of these isolates to the community has yet to be determined.

*Staphylococcus aureus* can also infect a variety of other mammals and birds. The more common natural infections include mastitis, synovitis, arthritis, endometritis, furuncles, suppurative dermatitis, pyemia, and septicemia. Staphylococcal mastitis in either a clinical or subclinical form may have considerable economic consequences in the dairy industry. *Staphylococcus aureus* subsp. *anaerobius* is the etiologic agent of an abscess disease in sheep, symptomatically similar to caseous lymphadenitis (De la Fuente et al., 1985). Hagan and Bruner’s *Microbiology and Infectious Diseases of Domestic Animals* (Timoney et al., 1988) may be referenced for further information on the nature of *S. aureus* infections in animals.

*Staphylococcus intermedius* is a serious opportunistic pathogen of dogs and may cause otitis externa, pyoderma, abscesses, reproductive tract infections, mastitis, and purulent wound infections. *Staphylococcus hyicus* has been implicated as the etiologic agent of infectious exudative epidermitis (greasy pig disease) and septic polyarthritis of pigs, skin lesions in cattle and horses, osteomyelitis in poultry and cattle, and occasionally associated with mastitis in cattle. *Staphylococcus delphini* has been implicated in purulent skin lesions of dolphins (Varaldo et al., 1988). *Staphylococcus schleiferi* subsp. *coagulans* is
associated with external auditory meatus of dogs (Igimi et al., 1990).

Although the coagulase-negative staphylococcal species constitute a major component of the normal microflora in humans, their role (especially that of *S. epidermidis*) in causing nosocomial infections has been recognized and well documented over the last two decades. The increase in infections by these organisms has been correlated with the wide medical use of prosthetic and indwelling devices and the growing number of immunocompromised patients in hospitals. Infectious processes may result from the introduction of endogenous staphylococci beyond the normal integumentary barriers. *Staphylococcus epidermidis* appears to have the greatest pathogenic potential and adaptive diversity. This species has been implicated in bacteremia, native and prosthetic valve endocarditis, osteomyelitis, pyoarthritis, peritonitis during continuous ambulatory dialysis, mediastinitis, infections of permanent pacemakers, vascular grafts, cerebrospinal fluid shunts, prosthetic joints, and a variety of orthopedic devices, and urinary tract infections including cystitis, urethritis, and pyelonephritis. Recent reviews have been published on the nature of human infections caused by *S. epidermidis* and other coagulase-negative species (Kloos and Bannerman, 1994; Rupp and Archer, 1994; Crossley and Archer, 1997). Nosocomial meticillin-resistant *S. epidermidis* (MRSE) strains became a serious clinical problem in the 1980s, especially in patients with prosthetic heart valves or who had undergone other forms of cardiac surgery (Archer and Tenenbaum, 1980; Karchmer et al., 1983). *Staphylococcus epidermidis* has also been occasionally associated with mastitis in cattle (Baba et al., 1980; Devriese and De Keyser, 1980; Holmberg, 1986).

Certain other coagulase-negative species have been associated with infections in humans and animals. *Staphylococcus haemolyticus* is the second most frequently encountered species of this group found in human clinical infections. It has been implicated in native valve endocarditis, septicemia, peritonitis, and urinary tract infections, and is occasionally associated with wound, bone, and joint infections. *Staphylococcus haemolyticus* has been occasionally associated with mastitis in cattle (Baba et al., 1980). *Staphylococcus caprae*, previously misidentified as *S. haemolyticus*, *S. hominis* and *S. warneri*, is widely distributed in human clinical specimens (Kawamura et al., 1998) and has been implicated in cases of infective endocarditis, bacteremia, and urinary tract infections. *Staphylococcus lugdunensis* has been implicated in native and prosthetic valve endocarditis, septicemia, brain abscess, and chronic osteoarthritis and infections of soft tissues, bone, peritoneal fluid, and catheters, especially in patients with underlying diseases. *Staphylococcus schleiferi* has been implicated in human brain empyema, osteoarthritis, bacteremia, wound infections, and infections associated with a cranial drain and jugular catheter. This species occurs less frequently than *S. lugdunensis* in the hospital environment and human infections. *Staphylococcus saprophyticus* is an important opportunistic pathogen in human urinary tract infections, especially in young, sexually active females. It is considered to be the second most common cause of urinary tract infections, such as acute cystitis or pyelonephritis, in these patients. This species can also produce in men urinary tract infections, which (unlike those in women) occur most commonly in the elderly with predisposing diseases of the urinary tract (Marrie et al., 1982a; Hovelius et al., 1984; Hovelius, 1986). *Staphylococcus saprophyticus* has occasionally been isolated from wound infections and septicemia (Marsik and Brake, 1982; Fleuret et al., 1987).

Several other coagulase-negative species have been implicated at low incidence in a variety of human infections. In most cases, patients with these infections had predisposing or underlying diseases that drastically altered their immune systems, and had also experienced surgery or intravascular manipulations. *Staphylococcus warneri* has been on occasion the etiologic agent of vertebral osteomyelitis, native valve endocarditis, and urinary tract infections in males and females. This species has been associated with mastitis in cattle (Devriese and Derby, 1979b; Devriese and De Keyser, 1980). *Staphylococcus simulans* has been associated with human chronic osteomyelitis, pyarthrosis, and bovine mastitis. *Staphylococcus felis*, a relative of *S. simulans*, has been isolated from clinical infections in cats, including external ear otitis, cystitis, abscesses, wounds, and other skin infections (Igimi et al., 1989). *Staphylococcus capititis* has been implicated in endocarditis, septicemia, and catheter infections. *Staphylococcus hominis* has been associated with human endocarditis, peritonitis, septicemia, and arthritis. Some of the earlier reports indicating an association of this species with infections were in error, owing to the misidentification of phosphatase-negative strains of *S. epidermidis* as *S. hominis*. *Staphylococcus cohnii* has been associated with urinary tract infections and arthritis. *Staphylococcus chromogenes*, a close relative of *S. hyicus*, is commonly isolated from the milk of cows suffering from mastitis, although its role as an etiologic agent is questionable (Devriese and De Keyser, 1980; Langlois et al., 1983; Watts et al., 1984). *Staphylococcus sciuri* subspecies has been iso-
lated from wound, skin, and soft tissue infections (Marsou et al., 1999).

Members of the *Macroccoccus* genus constitute a portion of the normal microflora of cattle, horses and ponies (Kloos et al., 1998a). *Macroccoccus caseolyticus* has been isolated from abscesses of slaughtered lambs (de la Fuente et al., 1992).

**Applications**

It has been known that Gram-positive and catalase-positive cocci play an important role in the ripening process of dry sausages (Lerche and Sinell, 1955; Niinivaara and Pohja, 1956). The predominant microorganism in fermented meat is *S. carnosus*, appearing in the early literature as *Micrococcus* (Schleifer and Fischer, 1982). For more than 50 years, *S. carnosus* has been used alone or in combination with lactobacilli or pediococci, as a starter culture for the production of raw fermented sausages. One of the main advantages of starter cultures in fermented food processing is that the fermentation and ripening process can be carried out under controlled conditions. In this way, food-poisoning and food-spoilage microorganisms can be suppressed, and the course of the fermentation process and its termination more reliably monitored. During the ripening process of dry sausage, *S. carnosus* exerts several desired functions (Liepe and Porobic, 1983; Götz, 1990). First, *S. carnosus* gradually reduces nitrate to nitrite and nitrite to ammonia. The advantage of this reaction is that the nitrate concentration is lowered, and that nitrite can combine with myoglobin to form nitrosomyoglobin, which gives the typical red color. As outlined above, nitrite is further reduced to the ammonia thus lowering the unbound nitrite concentration (Neubauer and Götz, 1996). Other advantages are development of characteristic flavor, lowering the pH moderately, and the capacity to reduce hydrogen peroxide produced by the catalase-negative lactobacilli, thus preventing odors.

Another application of *S. carnosus* is as an alternative host organism for the production of heterologous proteins or hormones. Transformation systems (Götz and Schumacher, 1987; Augustin and Götz, 1990) have been worked out and useful plasmid vectors have been constructed (Wieland et al., 1995; Peschel et al., 1996). With the aid of the lipase propeptide, an efficient secretion system has been worked out (Götz and Rosenstein, 2001b), which has been used successfully for secretion of heterologous proteins such as the human growth hormone (Sturmfels et al., 2001).

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