

**From the Department of Milk Sciences
Institute of Veterinary Food Sciences
Justus-Liebig-University, Giessen**

**Identification and Further Characterization of
Streptococcus uberis and *Streptococcus parauberis* Isolated
from Bovine Milk Samples**

**A Dissertation Submitted for the Acquisition of Doctoral Degree
of Veterinary Science
at
Faculty of Veterinary Medicine
Justus-Liebig-University, Giessen
Germany**

**Submitted by
Izhar ul-haq Khan**

Giessen 2002

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Supervisor: Prof. Dr. Ch. Lämmler

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With the Authorization of the Faculty of Veterinary Medicine
Justus-Liebig-University, Giessen

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*Dedicated to my
Beloved
Maternal Grandparents
and
Parents*

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1. Introduction

Mastitis is an inflammation of the mammary gland. Bovine mastitis usually arises as a result of an intramammary infection by bacteria. The signs may range from invisible abnormalities with a high rise in total somatic cell count to visible abnormalities in the milk with protein aggregates or clots accompanied by pain and swelling in the affected gland to production of a secretion which is composed solely of aggregated protein in a serous fluid. The inflammatory reaction and the increase in neutrophil numbers results in a lower rate of milk production and a gross deterioration of the quality of the secretion (DVG 1994; Wendt 1998). Mastitis remains economically the most important disease of dairy cattle throughout the world, with the three streptococcal species *Streptococcus uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* and the staphylococcal species *Staphylococcus aureus* being among the most significant pathogens involved (Fox and Gay 1993). *S. uberis* is an environmental pathogen responsible for a high proportion of cases of clinical, mostly subclinical mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the non-lactating period. Mastitis caused by *S. uberis* appears to be increasing in dairy cattle populations internationally. In particular *S. uberis* accounted for approximately 90% total cases of environmental streptococcal mastitis in heifers, within the first 5 days of lactation (Pankey et al. 1996). *S. uberis* differs from other mastitis-causing streptococci in that it can also be isolated from the udder surface, from other sites on the body of cows and also from the cow's environment. Therefore an udder infection with *S. uberis* is of major importance since it is poorly controlled by existing measures such as teat dipping and antibiotic dry cow therapy (Smith et al. 1985a; Hill 1988a).

Using DNA-DNA hybridization techniques Garvie and Bramley (1979) demonstrated the presence of two genotypes of *S. uberis*, designated types I and II. Comparative analysis of the 16S ribosomal RNA sequence of both subtypes additionally supported these findings and had been the basis of a proposal that subtype II should be designated as *Streptococcus parauberis*. However, the biochemical and serological characteristics of these two species are almost indistinguishable (Williams and Collins 1990). A molecular identification of both

species could be performed by analysis of restriction fragment length polymorphisms of the 16S rRNA gene (Jayarao et al. 1991; Jayarao et al. 1992; Lämmler et al. 1998a) or by the use of species-specific oligonucleotide probes (Bentley et al. 1993; Bentley and Leigh 1995).

The present study was designed to investigate and differentiate *S. uberis* and *S. parauberis* strains isolated from subclinical and clinical bovine mastitis from different locations in Hesse, Germany, on the basis of cultural, biochemical, serological and other phenotypic characteristics and by molecular methods using polymerase chain reaction technology. Additionally epidemiological relationships of the *S. uberis* isolates were investigated by macrorestriction analysis of their chromosomal DNA by pulsed-field gel electrophoresis.

2. Review of Literature

2.1 History and Taxonomy

The genus *Streptococcus* is found in a wide variety of human, animal and plant habitats. Streptococci are important in the dairy industry and as pathogens of animals and humans. The genus was first described by Rosenbach (1884) and its history had been reviewed by Wilson and Miles (1975) and Jones (1978).

In the last decades there had been several taxonomic analyses, leading to a comprehensive study of large parts of the genus *Streptococcus*. These findings were incorporated in varying degrees into taxonomic revisions. The most important contributions regarding the classification of streptococci on the basis of cultural, biochemical and serological characteristics were done by Andrewes and Horder (1906), Orla-Jensen (1919), Lancefield (1933) and Sherman (1937). The species *Streptococcus uberis* associated with bovine mastitis was first described by Diernhofer (1932). Slot (1958) investigated *S. uberis* also including the criteria mentioned by Sherman (1937). According to these studies *S. uberis* does not belong to genus *Enterococcus* but showed similarities with the pyogenic group of genus *Streptococcus*. Roguinsky (1972) compared some physiological properties of strains of *S. uberis* with those of seven other species found in the same habitat and concluded that *S. uberis* was a well-defined separate species. Facklam (1977) classified the genus *Streptococcus* into four major groups: *Streptococcus pyogenes*, *Streptococcus viridans*, *Streptococcus lactis* and *Enterococcus*. *S. uberis* was included in this study as “viridans” streptococci. Both studies used physiological tests and serology. The latter technique revealed several serological groups in strains, which physiologically belonged to a single species. In 1983, Bridge and Sneath conducted a numerical taxonomic study of strains of genus *Streptococcus* together with representatives of allied genera showing 28 reasonably distinct phenons. *S. uberis* was classified under paraprogenic species also including *Streptococcus dysgalactiae* and Lancefield groups R, S and T streptococci. Schleifer and Kilpper-Bälz (1984) and Schleifer (1985) performed a taxonomic revision of genus *Streptococcus* and classified this genus into three genera, i.e. *Streptococcus sensu stricto*, *Enterococcus* and *Lactococcus*. Later Schleifer and

Kilpper-Bälz (1987) further subdivided genus *Streptococcus* into three groups including pyogenic streptococci, oral streptococci and other streptococci. However, on the basis of cultural and biochemical characteristics as well as serological heterogeneity *S. uberis*, along with other streptococcal species, was categorized under other streptococci.

According to Hardie (1986) the taxonomy of *S. uberis* appears to be unsatisfactory. The species was biochemically and serologically heterogeneous. On the basis of DNA-DNA hybridizations Garvie and Bramley (1979) as well as Collins et al. (1984) demonstrated a genetic heterogeneity and the existence of two genotypes designated as type I and type II *S. uberis*. After comparative analysis of nucleotide sequences of 16S ribosomal-RNA of *S. uberis* type I and II Williams and Collins (1990) suggested for type II *S. uberis* the new species name *Streptococcus parauberis*.

The recent list of bacterial names also including *S. uberis* and *S. parauberis*, summarized by Euzéby (1997), are available on the internet ([www-sw.cict-fr/bacterio/details](http://www-sw.cict.fr/bacterio/details)).

2.2 Epidemiology

S. uberis, *S. agalactiae* (Lancefield's serogroup B), *S. dysgalactiae* (Lancefield's serogroup C), as well as streptococci of serogroup D, G, L, O and P are the main agents causing bovine mastitis (Hahn 1980).

The species *S. uberis* is most commonly found as a pathogen of the dairy cow throughout the entire lactating and non-lactating period. The most important reservoirs for infections of the mammary gland parenchyma with *S. uberis* appears to be the skin and the udder surface (Sweeney 1964; Cullen, 1966; Schalm et al. 1971; King 1981b). In addition, these bacteria can be isolated from numerous sites of the body including belly, lips, teats and urogenital tract (Sharma and Parker 1970), tonsils (Obliger 1954; Daleel and Frost 1967), rectum, rumen, nostrils, eye, poll, chest, sacrum, caudal folds and feces (Sweeney 1964; Cullen 1966; Sharma and Parker 1970 and Lerondelle 1985). Slot (1958) described the occurrence of

S. uberis in the intestinal flora of cattle by examination of the flora of feces from cows and calves.

S. uberis is commonly described as an environmental pathogen because it has a greater ability than other mastitis pathogens to survive and multiply in extramammary sites. *S. uberis* had also been isolated in large numbers from the straw bedding of housed cattle. These bacteria usually occur during the winter housing period and from the pasture grazed by infected cattle (Bramley 1982).

Hughes (1960) and Cullen (1966) described that a *S. uberis* mastitis is often associated with calving and also more prevalent during the winter months. This enhanced incidence of infection might be a reflection of the dirty environmental conditions which often prevail during that period. Sharma and Parker (1970) also suggested that seasonal and climatic changes are responsible for variation in the incidence of *S. uberis*.

Hughes (1960) analyzed milk samples taken from clinical cases of bovine mastitis. The incidence of infection had been considered in relation to the season of the year, the age of the infected animals and the stage of their lactations. This author demonstrated that *S. uberis* comprised 18% of all infections and showed a fluctuation from year to year. Most *S. uberis* infections occurred in the months of winter, while infection declined by the second half of summer. The peak incidence of infection recorded in relation to age was in the 5 to 6 year group. Cullen (1967) described that *S. uberis* could frequently be isolated from bovine skin. It survived best in the moist microclimate of the lips. Cullen (1967) suggested that the presence of *S. uberis* on the lips and the occasional occurrence in rectal swabs implied that *S. uberis* might also pass through the alimentary canal.

Sweeney (1964) and Cullen (1966) also examined seasonal trends. These authors demonstrated that an infection of milk with *S. uberis* occurred secondary to skin infections and that during the summer the level of skin infection fell to a very low level.

Cullen and Little (1969) isolated *S. uberis* from rumen and soil and suggested that lips, tonsils and rumen might consistently reinfect one another due to swallowing and eructation. Moreover, *S. uberis* was obtained more easily from the soil during the early part of the year than later and was found mainly in the

wettest part of the field, where the cows tended to congregate, probably contaminating the pasture, rather than the converse. Similar findings were reported by Sweeney (1964) and Cullen (1966).

In 1950, Neave et al. concentrated on subclinical infections of *S. uberis* in the dry period and indicated that the highest rate of new infections occurred during the first weeks of the dry period. In contrast, the experimental work carried out by Reiter et al. (1970) indicated that the susceptibility of the udder to a *S. uberis* bacterial infection was lower in the early dry period than later.

In 1986, Wilesmith et al. determined the incidence of clinical mastitis and the associated bacteria in dairy herds. *S. uberis* was the pathogen most frequently isolated from clinical cases which occurred in the dry period. The incidence increased with advancing age, with highest rates of recurrence. Similar epidemiological features of clinical mastitis in dry cows from a three year study were demonstrated by Francis et al. (1986). Bacteriological examinations were carried out on secretions from all clinical cases and it was reported that *S. uberis* was the predominant pathogen associated with dry period clinical cases as well as that the risk of clinical cases in the dry period was higher than during lactation. This difference in risk was more marked for *S. uberis* than for the other major pathogens. Clinical cases in dry cows associated with *S. uberis* were observed in every month with the highest incidence occurring in June. However, the incidence of clinical mastitis caused by other pathogens including *S. dysgalactiae*, *Staphylococcus aureus*, *Escherichia coli* and (*Corynebacterium*) *pyogenes* increased during the winter housing period (October to March), a peak occurring at the end of this period, while a secondary peak in incidence was recorded in August (Francis et al. 1986).

Cullen (1969) worked with lactating and nonlactating cows and reported that the rate of isolation of *S. uberis* in both groups was similar. On the other hand Sharma (1969) reported that the incidence of *S. uberis* infection was highest in winter and least in summer. The infection was found to be highest during the early lactation period, followed by mid and late lactation, and according to this author, least during the dry period. The incidence of udder infection increased as the cows advanced in age. Ward and Schultz (1974) reported a marked increase of dry

period streptococcal intramammary infection as the age of the cow increased. The majority of the streptococcal intramammary infections was caused by *S. uberis* followed by *S. agalactiae*.

Smith et al. (1985b) conducted a surveillance of environmental pathogens causing mastitis in dairy herds. Out of 59% streptococcal infections *S. uberis* were isolated in 31.5% of the cases. The rate was higher during the dry period than during lactation and increased progressively as parity increased. The rate of incidence was maximal during summer.

Lerondelle (1985) and Wilesmith et al. (1986) stated that *S. uberis* rarely gives rise to clinical mastitis. The infection often remains subclinical during a long period of time and in the absence of treatment this pathogen causes serious losses in milk production. According to these authors *S. uberis* is the principal cause of mastitis in dry cows. However, the infection is not directly influenced by the season, the stage of lactation or the breed of the animals.

Robinson et al. (1985) manifested that the rate of *S. uberis* mastitis was 72% higher in the partially treated control group (only selective dry-cow therapy) than in the fully treated control group (teat dipping and dry-cow therapy). Of the mastitis cases 40% clinical cases were recorded during the dry period or within 48 hours after calving and 38% clinical cases were found during lactation. The latter occurred in quarters subclinically infected with the same pathogen at calving. An increased susceptibility to a *S. uberis* infection associated with an early involution was reported by McDonald and Anderson (1981). These authors found that glands were more likely to become infected following intramammary inoculation of *S. uberis* as involution progresses. Wilson and Richards (1980) determined the prevalence of subclinical mastitis in British dairy herds. They studied 500 herds and recorded the prevalence of the various infections. *S. uberis* was found in 1.5% of the quarters, *S. agalactiae* in 3.4%, *S. dysgalactiae* in 1.1% and *Staphylococcus (pyogenes)* in 8.1%.

In 1983, Kruze et al. studied in vivo and post mortem cases and observed that *S. uberis* colonizes the bovine genital tract, particularly the deeper part of the vagina and cervix. The bacteria were recovered most frequently and in greatest

numbers immediately pre- and post partum, a period when most *S. uberis* mastitis occurs.

Zehner et al. (1986) conducted an experiment on different sterilized bedding materials under controlled conditions. These authors determined that the bedding materials vary in their ability to support the growth of different environmental pathogens. The growth was independent of the presence of feces, urine or other contamination. A rapid growth of *S. uberis* was seen in straw and recycled manure. However, some growth occurred in hardwood chips and less growth was observed in paper and softwood sawdust. These studies showed that clean, damp bedding might support bacterial growth.

Jayarao et al. (1999) conducted a study on lactating cows and reported that the incidence of *S. uberis* is high in cows with four or more lactations, compared with cows with 3 or fewer lactations. Regardless of lactation number the prevalence of *S. uberis* was highest before parturition, during early lactation and near drying off. These authors reported that *S. uberis* appears to be a major causative agent for subclinical mastitis in dairy cattle. However, Leigh (1999, 2000) reported that *S. uberis* is one of the streptococcal species which is responsible for a significant proportion of clinical bovine mastitis.

Barkema et al. (1999) studied the risk factors for the incidence rate of clinical mastitis in Dutch dairy herds. The study was conducted on the basis of two categories: variables associated with resistance to disease were feeding, housing and milking machine factors and variables associated with exposure were grazing, combined housing of dry cows and heifers and calving area hygiene. The incidence rate of *S. uberis* causing clinical mastitis was associated with factors related to housing, nutrition and machine milking, while the incidence rate of clinical mastitis caused by *S. dysgalactiae* and *S. aureus* was related to nutrition, milking technique and machine milking. In an additional study Barkema (1999) described similar factors related to the incidence of *S. uberis* clinical mastitis. Additionally, the author worked with two different groups of farmers and herds. The first group was recorded as “Clean and Accurate” and the other as “Quick and Dirty”. The relationship between these two groups and bulk milk somatic cell count category

was high. However, the relationship between the two groups and the incidence of clinical mastitis was weak.

Dynamics of *S. uberis* infections at a herd level were recently reported by Zadoks et al. (2001). The bacteriological cultures were routinely collected at 3 week intervals from all lactating animals. Additional samples were collected at calving, from cases of clinical mastitis, from dry cases, and after culling, respectively. During the 78 weeks of observatory period, 54 *S. uberis* infections were recorded. The majority of infections occurred during a 21 week period that constituted the disease outbreak. It was observed that the incidence rate was higher in quarters that had recovered from prior *S. uberis* infection than in quarters that had not experienced a *S. uberis* infection before. The incidence rate of *S. uberis* infection did not differ between quarters that were infected with other pathogens compared with quarters that were not infected with other pathogens. These authors suggested that contagious transmission might play a role in the outbreak of *S. uberis* mastitis.

Costa et al. (1998) determined the prevalence and identification of environmental pathogens causing mastitis in dairy herds. In addition, the influence of season, housing and management were evaluated. The most frequently isolated environmental pathogens were algae of the genus *Prototheca* (41.2%), *S. uberis* (21.1%), fungi (19.5%), *Enterobacteriaceae* (8.3%) and *Nocardia* sp. (6.6%). The occurrence of mastitis was not influenced by the herd size, use of dry cow therapy, or post milking teat dipping. A tendency for an increased occurrence of environmental mastitis during the months of September to February (hot and wet weather) was observed, suggesting a seasonal influence.

In 1985, Robinson et al. reported that *S. uberis* as a major pathogen was more frequently isolated from teat ends, which resulted from the fecal contamination of teats. Also flies might be important in spreading *S. uberis* infections, possibly with cow gut as source.

Mackey and Hinton (1990) analyzed the survival of *S. uberis* in feed and on straw with other streptococci and enterococci. The studies, carried out for one month, revealed that feed is not likely to be an important source of mastitis pathogens for cattle. It also proved to be an unsatisfactory environment for

S. uberis. On the other hand in straw all four other bacterial species *S. uberis*, *S. bovis*, *Enterococcus faecalis* and *Enterococcus faecium* survived comparatively.

Morea et al. (1999) isolated *S. uberis* with other *Streptococcus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Carnobacterium* and *Leuconostoc* species from mozzarella cheese. The identification of the bacterial population was performed by their physiological properties. The techniques allowed the identification of potential pathogens in a non-ripened cheese produced from raw milk.

2.3 Pathogenesis

2.3.1 Importance in Bovine Mastitis

S. uberis is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is the predominant organism isolated from mammary glands during the non-lactating period (Bramley 1984; Bramley and Dodd 1984; Oliver 1988).

Compositional changes in milk during mastitis reflect inflammatory changes in the udder. The status of the udder can be monitored by the indicators of inflammation. The first detectable change is the appearance of plasma proteins in milk as a consequence of increased permeability between the blood and milk compartments. Neutrophil leukocytes migrate into the milk due to various chemotactic stimuli. Electrolytes, such as sodium and chloride, also leak into milk and the pH of the milk approaches that of blood. The synthesizing capacity of the gland is suppressed. Pathological effects on the udder tissue vary with the type of the invading organism (Schalm 1977).

Adhesion to host cells has been shown to be an important first stage in the early pathogenesis of many bacterial diseases (Jones and Rutter 1972). It has been suggested that the adhesion of bacteria to the epithelia of the mammary gland may help to prevent removal of the pathogen from the gland during milking and may, therefore, play a role in determining the ability of bacteria to colonize this site and hence cause bovine mastitis (Frost et al. 1977; Harper et al. 1977). The ability of

S. uberis to adhere to cells from the bovine mammary gland has been shown by various authors. These investigations used cells which were derived by enzymatic digestion or mechanical disruption of the tissue (Opdebeek et al. 1988) or cells from immortalized lines (Mathews et al. 1994b). Roguinsky (1977) examined and compared the udder pathogenicity by experimental intramammary inoculation of *S. uberis* and *S. frequens* during the dry period. The studies showed that both species produced similar udder infections. Using *S. uberis* strains the author observed a notable difference between strains. In case of transmission of infection, *S. uberis* always strongly adhered to udder cells but some of them adhered very poorly. However, *S. frequens* strains failed to exhibit a particular adherence to udder cells.

Adherence of bacteria to host cells has been suggested as prerequisite for the colonization and establishment of infection. Thomas et al. (1992) reported about the use of explant cultures of bovine mammary tissue to investigate the hypothesis that adhesion to epithelium may be the first stage of pathogenesis. This was later supported by Almeida et al. (1993). These authors showed that adherence of strains of *S. uberis* seems to be mediated by collagen, fibronectin and laminin. Falkow (1991) described that in vivo *S. uberis* has an ability to invade bovine mammary epithelial cells which could result in protection from host defense mechanisms and from the action of most antimicrobial agents. This might be an important mechanism in the pathogenesis of *S. uberis* mastitis. Recently, Lammers et al. (2001) conducted a study on the specificity and efficiency of adhesion of bovine mastitis pathogens to bovine mammary gland cells and observed that, compared to other pathogens, *S. uberis* strains adhered mainly to cubic cells. The cubic cells could not express fibronectin. *S. uberis* cells bound fibronectin less efficiently, however, the adhesion of *S. uberis* cells seemed to be independent of fibronectin binding.

Ditcham et al. (1996) worked with monolayers of epithelial cells obtained by culturing of isolated secretory alveoli from the bovine mammary gland. These cells were used in bacterial adhesion assays. The ability of two strains of *S. uberis* to adhere to these cells was examined using a scanning electron microscope. The cultured monolayers consisted of two types of epithelial cells, one of which

possessed microvilli and another which exhibited only sparse or no microvilli. The more virulent *S. uberis* of the two strains used did not show any greater ability to adhere to the cultured cells than the other one. Unlike the ability to grow in milk and to avoid phagocytosis by neutrophils, the ability to adhere to host tissue did not appear to account for the differing virulence of the strains (Ditcham et al. 1996).

Thomas et al. (1994) evaluated the pathological findings of experimentally induced *S. uberis* infections in the mammary gland of cows. The authors observed that an acute inflammatory response involved an accumulation of large numbers of polymorphonuclear, neutrophilic leukocytes in the secretory acini.

Hill (1988b) reported that a primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain. In relation to this study Leigh et al. (1990) described that some strains were significantly more resistant to phagocytosis by bovine neutrophils following growth in the presence of milk, whey, casein or casein-derived amino acids. In connection to previous studies Hill et al. (1994) carried out an experiment on the pathogenesis of experimentally induced intramammary infection with *S. uberis*. These authors reported that neutrophils and specific opsonising antibody fail to form the major defense against infection with *S. uberis*. All three studies suggested that bacteria growing in the lactating mammary gland may elaborate a pathogenic determinant in form of an antiphagocytic factor.

Clinical and experimental data supported the concept, as documented by Eberhart 1982, Smith et al. 1985a, b, Oliver 1988 and Oliver and Sordillo 1988, that bovine mammary glands are susceptible to new intramammary infections during the physiological transition of the gland from lactation to involution and from involution to colostrogenesis. *S. uberis* grew well in mammary secretions of involuted glands and poorly in secretions during lactation.

Sordillo and Nickerson (1986) conducted an experiment on mice, in order to modify the susceptibility of mammary glands to *S. uberis* infection. Murine mammary glands were injected with pokeweed mitogen (PWM) prior to experimental bacterial challenge to accelerate involution and enhance antimicrobial mechanisms. The data indicated that PWM injection reduced the

numbers of *S. uberis* recovered when compared to controls. These authors suggested that PMN provided some protection against *S. uberis* mastitis by accelerating mammary involution, enhancing antimicrobial defenses, and facilitating a marked cellular response prior to bacterial challenge.

In one field experiment carried out by Grommers et al. (1985), the authors estimated and compared the duration of subclinical infections of *S. uberis* with *S. agalactiae*, *S. dysgalactiae* and *S. aureus* over a period of three and a half years. Of these infections the average duration of spontaneously eliminated infections for *S. uberis* was significantly higher (62%), while the average duration of infections persisting until drying off was relatively lower (36%) than for the other three species, respectively. The authors concluded that the respective analysis was of value for estimating new infection rates and for selecting quarters for dry cow therapy.

Doane et al. (1987) inoculated *S. uberis* intramammarily in lactating cows naturally colonized with *Corynebacterium bovis*. A clinical mastitis developed in 3 days, the mastitis was acute, showed high rectal temperature, significant increase in somatic cell count and a decrease in milk production. There were no differences observed in the severity of *S. uberis* mastitis in quarters colonized with and without *C. bovis*.

In 1993, Leigh and in 1997, Kitt and Leigh studied nutritional requirements of *S. uberis* and analyzed that *S. uberis* is auxotrophic for between 10 and 13 amino acids, 8 amino acids were commonly required by all the strains. It has also been postulated that early in the pathogenesis, prior to the induction of an inflammatory response, the growth of *S. uberis* is facilitated by the ability to hydrolyze host proteins. However, *S. uberis* does not hydrolyze protein directly. In a chemically defined medium in which a single essential amino acid was omitted, the inclusion of intact alpha, beta and kappa bovine casein failed to restore growth. In the absence of certain amino acids, growth of *S. uberis* can be restored by the inclusion of plasmin-hydrolyzed caseins, thus demonstrating that acquisition of some essential nutrients may be achieved by this route.

In 1991, Oliver demonstrated an influenced growth of Gram-positive mastitis pathogens in mammary secretions by induced mammary inflammation at

cessation of milking, particularly during early involution. The author performed an intramammary infusion of lipopolysaccharides (LPS) at cessation of milk. The data indicated that infusion of LPS did not alter the growth of mastitis pathogens in mammary secretions during the nonlactating period. According to these studies *S. uberis* and *S. agalactiae* grew better in mammary secretions obtained during involution than in secretions collected during the late or early lactation, the growth of *S. uberis* was high during physiologic transitions of the udder. *S. aureus* grew well during late lactation and *S. dysgalactiae* grew well at all time periods.

Fang et al. (1998) conducted a study on milk samples from mammary glands challenged with *S. uberis* and from unchallenged mammary glands. The samples were selected for analysis of bacterial growth, antibody response and lactoperoxidase activity. All challenged mammary glands became infected with *S. uberis* and had an elevated somatic cell count in milk during the first week after challenge. In vitro growth of the homologous challenge strain and a heterologous strain of *S. uberis* was significantly lower in milk from challenged mammary glands than in milk from control mammary glands. Specific antibodies increased in challenged glands whereas lactoperoxidase activity varied among cows and among different samples over time and did not contribute to the decreased growth of *S. uberis*. The decreased growth of *S. uberis* in milk from challenged mammary glands in comparison to milk from control mammary glands resulted from the interaction of antibodies with complement components.

In 1988, Sobiraj et al. examined and analyzed the clinical and bacteriological incidence of mastitis in first lactating heifers during and shortly after parturition. The colostrum samples of udders of 100 heifers were investigated for clinical changes during parturition. According to the data obtained, 35 heifers showed clinical changes of the quarters and/or colostrum changes. The milk contained blood clots, blood-mixed milk, color variation particularly due to fibrin flakes. According to the data observed by these authors, 14 of the animals suffered from a cellulitis-like (high grade variation in milk appearance including painful inflammatory and oedematous swelling of the mammary gland) mastitis, while 13 animals had an acute catarrhalic mastitis and 8 heifers showed acute galactophoritis. Beside this group 24 animals apparently healthy during parturition

failed to show the clinical signs of inflammation at least until the end of that study. The isolated bacteria included facultative and obligatory pathogenic species, 70% were Gram-positive cocci (i.e. *S. uberis*, *S. dysgalactiae*, *S. agalactiae*, *S. aureus* and other staphylococci) versus 30% of Gram-negative bacteria including *E. coli*, *Klebsiella* and other coliform species. According to these studies *S. uberis* was found in 39 of 58 samples. These authors suggested that *S. uberis* is one of the most prevalent mastitis causing streptococcal species in lactating heifers. Moreover they suggested that the varying degree of exposition to stress and the individual ability of the animal sub partu seems to be of far more importance for both onset and severity of mastitis.

The clinical signs and changes in the milk compositions mostly developed later in experimental streptococcal and staphylococcal mastitis than in coliform mastitis (Newbould and Neave 1965; Harmon et al. 1976; Higgs et al. 1980; Anderson et al. 1985). However, according to Franklin et al. (1984) experimental infection induced by *S. uberis* seems to be more delayed than with the other organisms investigated. On the basis of these studies Pyörälä and Mattila (1987) studied the inflammatory reaction in an experimental mastitis induced in nine lactating cows with *S. uberis*, *S. dysgalactiae* and *S. aureus*: each group consisted of three cows. One quarter of each cow was inoculated with organisms, the co-lateral one remained untreated and served as control. Inflammatory changes were monitored by measurements of somatic cell count, N-acetyl- β -D-glucosamidase and antitrypsin activity. The authors observed the clinical signs and the increase in the levels of the inflammatory parameters occurring 24 hours after inoculation. In these studies *S. aureus* caused more severe inflammatory changes than did streptococci. The inflammation declined slowly after the infection of the udder had been cleared. Whey was found to stimulate bacterial growth later in the course of inflammation in all experimentally infected quarters. The clinical signs, 36 h after challenge, were accompanied by an increase in milk content of the inflammatory markers such as somatic cell count, local and systemic clinical signs and NAGase enzyme activity. The latest response noted 36 h post-inoculation was in one quarter infected with *S. uberis*. The clinical signs were in most cases moderate or mild. Body temperature became elevated transiently but the appetite usually remained

unaffected. The infused quarter became more or less swollen and tender and clots were found in milk. Systemic signs disappeared within 12 hours without any treatment. Local signs subsided gradually during a somewhat longer period. Clinical mastitis was observed in 6 of the 9 experimentally infected cows. The time of the onset of visible signs corresponded with the above mentioned reports.

Numerous studies have been conducted to determine the dynamics of polymorphonuclear neutrophil (PMN) migration (diapedesis) across the epithelial lining into the infected lumen of various organs in several species (Ackerman et al. 1996; Agace 1996; Liu et al. 1996). Because of the complexity of these organ systems, monolayers of epithelial cells and isolated PMN had been used to determine the factor effecting PMN diapedesis. In relation to these studies Smits et al. (1998) studied the expression of PMN adhesion receptors such as CD11b/CD18 and diapedesis by PMN before and after an experimentally induced *S. uberis* mastitis. Both quarters of the left half of the udders of five midlactation cows were inoculated with *S. uberis*. Clinical signs of an inflammatory reaction and leukocyte influx were observed 24 h after challenge. In vitro, the epithelial cell monolayer and blood were used to measure diapedesis of PMN and expression of adhesion receptors, respectively. The data indicated that CD11b/CD18 adhesion receptors on blood PMN obtained were increased 24 h after *S. uberis* challenge, while diapedesis across secretory epithelial cells was depressed. These authors suggested that in *S. uberis* mastitis adhesion receptors factors other than CD11b/CD18 could be involved in PMN diapedesis.

According to Almeida et al. (2000) *S. uberis* has an ability to internalize into bovine epithelial cells, while inhibitors of F-actin microfilament polymerization inhibited the internalization. The authors analyzed and reported that inhibitors of eukaryotic cell tyrosine protein kinase (TPK) and protein kinase C (PKC) could also significantly reduce the internalization of *S. uberis* into mammary epithelial cells.

In association with mastitis King (1981a) reported that *S. uberis* also causes endometritis, endocarditis and abortions in cattle whereas in bulls a vesiculitis case was reported by Willems et al. 1962. In this study *S. uberis* was isolated from semen which contained many leucocytes.

2.3.2 Occurrence in Infections of Other Animals

S. uberis has also been reported as an important cause of mastitis in sheep (Pisanu and Manca 1964; Mettler 1986). In both studies *S. uberis* was isolated from sheep with clinical mastitis. In one of the sheep herds 35% of the animals became affected with mastitis after the introduction of mechanical milking. The mastitis appeared to be different from the subclinical forms caused by *S. uberis* among the cattle on the same farm. Pisanu and Manca (1964) suggested that a different source has been involved in the spread of the disease. *S. uberis* as causative agent was identified and confirmed by biochemical and serological tests. In further experiments the isolated strains caused, when experimentally injected into the udder of healthy sheep, a mastitis clinically identical to the naturally occurring mastitis.

In 1971, Jeleu et al. described cases of metritis, arthritis and parametritis caused by *S. uberis* in dams.

Deibel et al. (1964) and Hahn (1980) reported about the occurrence of *S. uberis* in the urogenital tract and lymphnodes of pigs, in mammary glands of mares and in the oral cavity of dogs.

A single isolation of *S. uberis* from a scimitar-horned oryx (*Oryx dammah*) has recently been reported by Chai (1999). This bacterium was cultured from endocarditis lesions which lead to a fatal congestive heart failure.

S. parauberis (formerly *S. uberis* genotype II) was previously described as a new species (Williams and Collins 1990). Doménech et al. (1996) reported about the isolation of *S. parauberis* from diseases in fish. The isolation was performed from juvenile and adult turbot. The disease occurred throughout the year, although the clinical signs were more severe and the mortality rate slightly higher during summer. The species was isolated from liver, kidney and spleen of diseased turbot. The mortality rate was estimated between 0.1 and 5% and was accompanied by high economic losses because of the pronounced loss of weight of affected turbot.

2.3.3 Occurrence in Infections of Humans

Facklam (1977) reported about the isolation of *S. uberis* from cases of endocarditis and urogenital infections in humans. In a study investigating 1227 “Viridans” streptococci from human sources seven strains were classified as *S. uberis*, two isolated from blood, two from body fluids, two from urogenital infections and one from dental plaque.

Rabe et al. (1988) studied the prevalence of viridans group streptococci in the female genital tract. The isolates included *S. uberis* (0.2%), followed by *S. intermedius* (13%), *S. acidominimus* (6%), *S. constellatus* (5%), *S. sanguis* II (4%), *S. mitis* (2%), *S. salivarius* (2%), *S. morbillorum* (2%), *S. sanguis* I (1%) and *S. mutans* (0.2%).

A single case of a hepatic abscess of a 61 year old cattle man was reported by Sanchez et al. (1991). The patient had deep abdominal right hypochondrial pain on palpation. A mass was observed after ecography in the right hepatic lobe. After drainage of 200 ml turbid and smelly liquid *S. uberis* could be cultivated by using conventional tests.

Bouskraoui et al. (1999) reported about the isolation of *S. uberis* from a case of endocarditis. *S. uberis* was isolated from an 11 month old infant hospitalized with a high rise of temperature. The blood cultures were confirmed on the basis of biochemical and serological characteristics and by antibiotic sensitivity tests. The isolates were remarkably sensitive to penicillin-G and amoxicillin. On the basis of previous reports and phenotypic characteristics the causative agent was identified as *S. uberis*.

2.4 Virulence Factors and Enzyme Activities

Since antimicrobial treatment is generally ineffective in treating *S. uberis* mastitis, the development of control measures must be based on an understanding of virulence factors and antigens involved in invasion and possibly protection of the mammary gland.

2.4.1 Hyaluronic Acid Capsule

Two general mechanisms have been proposed to account for the role of capsular polysaccharides in the resistance of bacteria to phagocytosis. First, the capsule may prevent the binding of opsonic factors such as antibody or complement to the bacterial cell (Horwitz and Silverstein 1980). Second, it may be permeable to opsonic factors which bind beneath the capsular surface so that the capsule presents a barrier that prevents subsequent contact between bound opsonin ligands and their receptors on the phagocytic cell (King and Wilkinson 1981). Therefore the capsule acts as a physical barrier to the efficient attachment of either opsonin or the phagocyte and the bacterium.

In addition, the capsule protects bacteria from phagocytosis by masking surface antigens, by causing consumption of complement fractions, by altering complement binding to the bacterial surface or by blocking receptors on the surface of the phagocyte. Bacterial capsule is considered a virulence factor primarily because of its antiphagocytic role. This can influence the establishment of infection (Verbrugh et al. 1979; Densen and Mandell 1980; Wilkinson 1983; Spitznagel 1983).

Hill 1988a, Leigh and Field 1991, Mathews et al. 1992a and Mathews et al. 1992b isolated encapsulated *S. uberis* from bovine mammary secretions. Almeida and Oliver (1992) indicated that the *S. uberis* capsule is composed primarily of hyaluronic acid. These authors suggested that the hyaluronate capsule seems to be a major virulence factor of *S. uberis*. The ability to alter the resistance of *S. uberis* phagocytosis in vitro has facilitated the comparison of individual strains which exhibit phagocytic resistant and susceptible phenotypes. This has shown that the production of a hyaluronic acid capsule correlates well with the ability to resist phagocytosis by neutrophils. If this layer is removed from the bacterial cell by treatment with hyaluronidase, and providing re-formation of the capsule is prevented by inclusion of hyaluronidase in the bactericidal assay, there is a significant reduction in the ability of *S. uberis* to resist phagocytosis.

Leigh and Field (1993) conducted a study in which they examined both the encapsulated and unencapsulated phenotypes of individual strains of *S. uberis*.

Both phenotypes bind equal quantities of antibody. In each case this bound antibody was intact, was in the correct orientation and presented the Fc terminus for interaction with immunoglobulin receptors on the neutrophil. According to these studies the capsular layer of *S. uberis* does not deter immunoglobulin from binding to the bacterium, nor does the bacterium appear to possess mechanisms by which bound immunoglobulin is cleaved to prevent its interaction with the neutrophil. Furthermore, the capsule structure did not act as a significant barrier for the interaction between the bound immunoglobulin and receptors on the neutrophil. Pre-opsonised capsular and non-capsular phenotypes of individual strains of *S. uberis* were phagocytosed equally by bovine macrophages. The data suggested that resistance to phagocytosis by neutrophils occurred despite of the presence of potentially opsonic immunoglobulin bound to the bacterium. The presence of the capsule on *S. uberis* specifically determined a resistance to opsonophagocytosis by neutrophils but not macrophages. Incubation of bovine neutrophils with capsule preparations obtained from phagocytic resistant strains of *S. uberis* resulted in the lysis of the phagocytic cell. However, at lower concentrations lysis did not occur but the ability of such cells to phagocytose bacteria was impaired. It was concluded that *S. uberis* is capable of producing a component which can be isolated from the capsular matrix and which possibly mediates its resistance to phagocytosis by neutrophils via a toxic effect on the phagocyte itself.

According to Paape and Guidry (1977) intramammary infections by encapsulated strains of *S. uberis* inhibited the already depressed phagocytic function of the mammary macrophages, resulting in an increased rate of new intramammary infections by encapsulated and non-encapsulated strains of *S. uberis* during the non-lactating period.

Hill (1988b) investigated the pathogenicity of two strains of *S. uberis*. The encapsulated strain was more pathogenic and more resistant to neutrophil phagocytosis than the non-encapsulated strain. Similarly Anzai et al. (1999) determined the pathogenicity and resistance of *S. equi* strains with different levels of capsule expression and compared the encapsulated and non-encapsulated status. These authors observed that the hyaluronic acid capsule reduced phagocytosis of

S. equi by neutrophils. Phagocytosis of encapsulated strains was increased after treatment with hyaluronidase whereas the non-encapsulated strain was sensitive to phagocytosis in the presence or absence of hyaluronidase, concluding that encapsulated strains were more resistant to phagocytosis as compared to the non-encapsulated strains.

According to a report by Rather et al. (1986) and Mathews et al. (1991b), subculture and prolonged storage may result in a complete loss in expression of virulence factors by many bacterial organisms. In addition, in vitro conditions can influence the ability of microorganisms to express capsule (Watson and Watson 1989; Leigh and Field 1991). On the basis of these reports Mathews et al. (1994a) determined and evaluated the occurrence of encapsulated *S. uberis* isolated from bovine mammary secretions and the influence of cultural conditions on expression of capsule, in order to investigate the pathogenicity of *S. uberis*. Strains of *S. uberis* were assessed by India ink for expression of capsule and evaluated under four different conditions. The number of strains expressing capsule decreased greatly after passage and following storage. Strains were also cultured in various media to determine the influence of medium components on capsule expression. The media supplemented with either serum or egg yolk enhanced the size of capsule expressed as compared to the other supplemented media. Additionally, Leigh and Field (1991) reported that supplementation of growth medium with milk whey, casaminoacids or casein increased the ability of some strains of *S. uberis* to resist phagocytosis and killing.

Almeida and Oliver (1992) carried out a study on non-opsonized encapsulated and non-encapsulated strains of *S. uberis*. The strains were incubated with bovine mammary macrophages and the percentage of phagocytosis was studied. In these studies 75% of macrophages ingested non-encapsulated organisms with a killing rate of 75%. In contrast, 48% of macrophages ingested encapsulated bacteria with a killing rate of 35%. When strains were opsonized with homologous antiserum, differences were detected in the percentage of phagocytosis (84% vs. 48%) and intracellular killing (52% vs. 35%) of the encapsulated strain only. These effects were partially abolished when antiserum was absorbed with purified capsular material, or when macrophages were pre-

treated with purified capsular material. Electron microscopy of mammary macrophages incubated with the encapsulated strain of *S. uberis* showed the microorganism in contact with the macrophage cell membrane without signs of membrane activation. In contrast, the non-encapsulated strain induced formation of pseudopods and membrane ruffling. It was observed that encapsulated *S. uberis* were more resistant to phagocytosis than non-encapsulated *S. uberis* and that antibodies against capsule enhanced phagocytosis of encapsulated bacteria.

Ward et al. (2001) determined the contribution of the capsule in conferring resistance to phagocytosis through the disruption of *hasC*-like gene directly involved in the assembly of the hyaluronic acid capsule. The acapsular phenotype of *S. uberis* displayed markedly reduced resistance to phagocytosis. These findings again support the correlation of the production of the hyaluronic acid capsule with resistance to phagocytosis by neutrophils of this bacterium.

2.4.2 CAMP Factor

The influence of staphylococcal β -lysin and exosubstances of primarily non-hemolytic streptococci lead in the zone of β -toxins from *S. aureus* to a complete zone of haemolysis. The lytic principle first described by Christie et al. (1944) is called CAMP phenomenon. The classical CAMP phenomenon with the typical half moon forming hemolytic zones was demonstrated on cattle or sheep blood agar plates. The streptococcal culture was streaked thereby in the right angle to a β -hemolytic *S. aureus* culture. After an incubation time of 18-24 hours at 37°C a half moon forming zone of complete lysis formed out within the area of the incomplete β -hemolysis of *S. aureus* (Hahn 1980). The CAMP factor of *S. agalactiae* was shown to be a thermostable protein with a molecular weight of 23.5 kDa and an isoelectrical point at pH 8.3 (Bernheimer et al. 1979). According to Jürgens et al. (1985) the CAMP factor was a protein with a molecular weight of 25 kDa and an isoelectrical point at pH 8.9. The β -toxin of *S. aureus* is a sphingomyelinase which alters the sphingomyelin of the erythrocyte diaphragm to ceramide phosphate (N-Acyl-sphingosylphosphate). The linkage of the CAMP factor to the ceramide phosphate caused a disorganization of the bilayer lipid of the

erythrocytes and led in such a way to the hemolysis of the erythrocytes (Bernheimer et al. 1979). The formation of the CAMP factor of group B streptococci depended on the composition of the growth medium. Of importance was both glucose and the maltose concentration. Both additives caused an increased formation of the CAMP factor (Huser et al. 1983; Tapsall and Philips 1985). After investigations of Lämmler et al. (1984), positive CAMP reactions developed within 8 hours after addition of maltose or glucose in a final concentration of 0.04%. A concentration of more than 0.64% maltose or glucose, however, prevented the formation of the CAMP reaction. The CAMP test is commonly used for identification of *S. agalactiae*. A β -toxin was shown for *S. aureus* and also for *S. intermedius* (Lämmler and Blobel 1987a). A certain error rate could be explained by the fact that streptococci of other serological groups likewise demonstrated a CAMP phenomenon. According to Kunter (1967), a CAMP reaction could also be observed for streptococci of serological groups E, C, P and L. Thal and Obiger (1969) observed positive CAMP reactions for streptococci of serological group U, and Lämmler et al. (1987) and Gürtürk and Lämmler (1990) for canine group G streptococci. According to Fraser (1962), *Pasteurella haemolytica* also showed synergistic haemolytic reactions with the β -toxin of *S. aureus*. Also substances of some coagulase negative staphylococci, as well as cultures of *Rhodococcus equi* and *Corynebacterium renale* showed such a similar CAMP phenomenon (Lämmler and Blobel 1987b). In addition, a positive CAMP reaction could be seen for cultures of *S. uberis* (Hahn 1981). The CAMP phenomenon of *S. uberis*, in contrast to the CAMP phenomenon of *S. agalactiae*, could not be demonstrated with the exotoxin of *Corynebacterium pseudotuberculosis* in place of the β -toxin of *S. aureus*. In the zone of the β -toxin the exosubstances of *S. agalactiae* and *S. uberis* caused a clear hemolysis. In the exotoxin zone of *C. pseudotuberculosis* a comparable zone of complete hemolysis could only be observed with *S. agalactiae* (Skalka et al. 1979). Both exosubstances of *S. uberis* and *S. agalactiae* were immunogenic and reacted specifically with their homologous antisera in agar gel diffusion (Skalka et al. 1980). After intraperitoneal application of the exosubstances of *S. uberis* and *S. agalactiae*, rabbits and mice reacted with spasms, vibration cramps and high-grade dyspnoea

within 2 minutes. The mice seemed to be insensitive compared to rabbits. The lethal dose was 45 times higher for the mouse than for the rabbit. In both animal species the application of sublethal doses led to the formation of neutralizing antibodies. After handling with formaldehyde both factors lost their lethal effect, as well as their synergistic hemolytic activity with the β -toxin of *S. aureus* (Skalka and Smola 1981). The nucleotide sequence of the *S. uberis* CAMP factor gene was determined by Jiang et al. (1996).

2.4.3 Hyaluronidase

The formation of the enzyme hyaluronidase has been described for streptococci of serological groups A, B, C, G, H, as well as L (Köhler 1963) and also for *S. uberis* (Schaufuss et al. 1989a; Christ and Lämmle 1992; Matthews et al. 1994 and Oliver et al. 1998). The hyaluronidase of *S. uberis* proved to be a protein with a molecular weight of 54 kDa and an isoelectrical point at pH 4.9. Hyaluronidases are extracellular proteins that split hyaluronic acid, an acidic mucopolysaccharide, into even-numbered oligosaccharides (Gerlach and Köhler 1972). The hyaluronic acid is composed of glucuronate and N-acetylglucosamine and is involved as extracellular material of the connective tissue in the structure of the intercellular basic substance of humans and animals and thus responsible for the connection of the individual cells in fabrics (Weide and Aurich 1979). Because of its high water permeability the hyaluronic acid is of importance for the osmoregulation (Landis and Pappenheimer 1963). With extracellular defense reactions hyaluronic acid plays a role as inhibitor of the migration of the leukocytes (Forrester and Wilkinson 1981), and a role in phagocytosis (Forrester and Balazs 1980). The reduction of the hyaluronic acid by hyaluronidase led to an increase of the fabric permeability. This facilitated the penetration of bacteria into the fabric. For this reason Duran-Reynals (1942) proposed the term spreading factor for hyaluronidase. Hyaluronidases work as antigens causing an antihyaluronidase reaction. After an infection with pathogen, which are capable for the production of hyaluronidase, specific antibodies could be observed in the serum (Jawetz et al. 1977). Isolated hyaluronidase also found a therapeutic application. After injections

of therapeutics in fabrics their propagation and absorption could be facilitated by hyaluronidase (Jawetz et al. 1977). According to Schaufuss et al. (1989b) and Matthews et al. (1994a), the production of hyaluronidase by *S. uberis* could facilitate the penetration of these bacteria into the tissue of the mammary gland and might play a role in pathogenicity.

2.4.4 Neuraminidase

A neuraminidase had been described for streptococci of the serological groups A, B and C (Hayano and Tanaka 1968), for streptococci of serogroups E, F, G, H, K, L, M, N and O as well as for *S. viridans*, *S. salivarius*, *S. sanguis* and *S. uberis* (Müller 1972). The enzymatic effect of this group of enzymes is based on the splitting of N-acetylneuraminic acid. These are β -ketosidic bound to oligosaccharides, gangliosides or glycoproteins (Aminoff 1961). To open for attacks by neuraminidase, a free OH group in C4 position of the acetylneuraminic acid seems to be responsible (Schauer and Faillard 1968). The neuraminidase of streptococci can be divided into 2 enzyme types. The first type was demonstrated for streptococci of serological group K, the second type with other streptococcal groups (Hayano et al. 1969). The neuraminidase of the K *Streptococcus* was able to split off neuraminic acid from the salivary mucin of cattle and from N-acetylneuraminyllactose of the bovine colostrum (Hayano and Tanaka 1968). The other streptococcal neuraminidase type set free neuraminic acid of the bovine salivary mucin (Hayano and Tanaka 1969). This neuraminidase type causes no release of neuraminic acid from N-acetylneuraminyllactose. An effect of neuraminidase as streptococcal antigen was described by Hayano and Tanaka (1968). Antibodies against neuraminidase of streptococci of serological groups A, B and E reacted exclusively with the homologous neuraminidases, while production of antibodies against G, C, and L streptococcal neuraminidases neutralized the neuraminidase activity of all 3 serogroups. Furthermore, interactions of antibodies against the neuraminidase of *S. sanguis* were observed with the neuraminidase of *S. uberis* (Hayano et al. 1969). Bacterial neuraminidases had also been described for *Vibrio cholerae* (Müller and Lütticken, 1974),

Corynebacterium diphtheriae (Müller 1976) and *Pasteurella multocida* (Müller 1971). *P. multocida* formed increased neuraminidase in unfavorable growth conditions. This could indicate the significance of the bacterial neuraminidase as “emergency mechanism” (Müller 1971). The pathogenic significance of the neuraminidase of streptococci is not yet exactly clarified. Neuraminidases of cariogenic bacteria such as *Pneumococcus*, *Bacterioides* species, *Fusobacteria* and *Bifidobacteria* facilitated the attaching of the bacteria to the teeth and a following germ invasion of the salivary mucins over a viscosity degradation. The neuraminidase of *Vibrio cholerae* seems to play a role in the formation of specific receptor sites for enterotoxins (Müller 1974, 1976).

2.4.5 Streptokinase

The ability of bacteria to grow in mastitic milk is enhanced by the presence of the caseinolytic enzyme plasmin (Mills and Thomas 1981). The transformation of plasminogen to plasmin requires plasminogen activators, which are known to occur in blood plasma and animal tissues (Collen 1980). Plasmin is a potent serine proteinase that has an important function in physiological processes in mammals, such as degradation of extracellular matrix proteins, blood clot dissolution (fibrinolysis), cellular migration, and for cancer metastasis Lottenberg et al. (1994).

Plasminogen, the blood-borne zymogen of plasmin, has two physiological activators. These activators are a tissue-type plasminogen activator and a urokinase-type plasminogen activator. These activators are themselves serine proteinases and activate plasminogen by cleavage of a single peptide bond. However, in addition to these two physiological plasminogen activators, several pathogenic microorganisms have developed plasminogen activators, which enable them to exploit host plasmin activity. The generation of plasmin activity assists the microorganism in proteolytic breakdown of fibrin and extracellular matrix proteins, which, in turn facilitates the bacterial penetration of normal tissue barriers and ultimately facilitates bacterial colonization of deep tissue sites (Boyle and Lottenberg 1997). Bacterial plasminogen activators include the enzyme

streptokinase, produced by a variety of pathogenic streptococcal species, and the enzyme staphylokinase, produced by *S. aureus*. Due to its fibrinolytic potential, streptokinase is currently used as a thrombolytic therapy drug. Streptokinase and staphylokinase have unique but slightly different mechanisms of plasminogen activation. Streptokinase and staphylokinase form 1:1 stoichiometric plasminogen activator complexes with plasminogen and plasmin, respectively. Streptokinase induces a conformation of the serine proteinase domain of plasminogen, which exposes the active site of proteinase without prior proteolytic cleavage, thereby providing the streptokinase-plasminogen complex with what has been called “virgin” enzyme activity (Reddy and Markus 1972). In contrast, the staphylokinase-plasminogen complex is proteolytically inactive but it can be transformed into the active staphylokinase-plasmin complex by activation with plasmin (Collen et al. 1993).

For a mastitis-inducing pathogen like *S. uberis*, the production of a plasminogen activator could be of importance in two ways. In addition to the generation of plasmin activity needed for degradation of extracellular matrix proteins and subsequent colonization, the activation of endogenous plasminogen present in milk would lead to hydrolysis of milk proteins and, thereby, liberation of peptides from which *S. uberis* could obtain essential amino acids (Kitt and Leigh 1997).

Some bacteria that produce plasminogen activators also produce plasmin(ogen) surface receptors. The binding of plasmin(ogen) to these receptors equips the bacteria with host-derived plasmin activity, and at the same time the receptors shield the bound plasmin from physiological inhibitors (Kuusela and Saksela 1990; Kuusela et al. 1992). Leigh and Lincoln (1997) reported that *S. uberis* has also an ability to bind bovine plasmin following cultivation in the presence of plasminogen. The authors concluded that the activation of plasminogen by a plasminogen activator is required prior to binding of plasmin by *S. uberis*. The acquisition of this activity might have effects on the pathogenesis of infection and induction of mastitis in the mammary gland. The binding of plasminogen to *S. uberis* was further characterized by Lincoln and Leigh (1998). These authors demonstrated that the plasmin:bacterium interaction was temporal

and mediated via the lysine binding sites of plasmin in a manner which leaves the active site of plasmin accessible for interaction with substrates.

In 1991, McCoy et al. manifested that streptokinases isolated from different strains of streptococci possess an intrinsic species specificity for their target plasminogen molecules that parallels the host range of the microorganisms. A bovine plasminogen activator designated as streptokinase of the bovine pathogen *S. uberis* was purified by Leigh (1993). According to this author certain streptococci, including *S. uberis*, are capable of producing streptokinase, which activates plasminogen to plasmin and it has been postulated that activation of plasminogen by streptokinase facilitates bacterial colonization during the very early stages of infection in the lactating gland by promoting the release of nutrients. In 1993, Leigh and Field speculated that streptokinase which activates bovine plasminogen might be an essential virulence factor of *S. uberis* allowing its rapid growth in the bovine mammary gland.

The streptokinase activity associated with the plasminogen activator secreted from *S. uberis* was different from that of *S. pyogenes* (Lancefield group A) and *S. equisimilis* (Lancefield group C) strains, as it activated bovine but not human plasminogen. It also differed from Lancefield group E streptokinase activity by not activating porcine plasminogen (Leigh 1993). In 1979, Casetellino reported that streptokinase from *Streptococcus equisimilis* (Lancefield group C) activates human and feline plasminogen, whereas the streptokinase of a Lancefield group E *Streptococcus* activated porcine plasminogen (Ellis and Armstrong 1971). Because of the differences in structure and substrate specificity the plasminogen activator of *S. uberis* was, according to Leigh (1994), distinct from other bacterial proteins which are capable of activating mammalian plasminogen. Instead of streptokinase these authors designated this molecule as plasminogen activator *uberis* (PauA). In 1998, Leigh et al. described a second novel plasminogen activator, derived from the bovine mastitis-inducing pathogen *S. dysgalactiae* that also showed specificity to bovine plasminogen. In the studies of Rosey et al. (1999) the *S. uberis* plasminogen activator gene *pauA* was sequenced.

Moreover, Johnsen et al. (1999) sequenced an additional plasminogen activator gene of *S. uberis* which the authors designated as streptokinase gene *skc*.

The authors showed in contrast to Leigh (1994) that this plasminogen activator was related to the already known streptokinases. In this relation Johnsen et al. (2000) investigated and demonstrated the kinetic mechanism of the plasminogen activation mediated by this novel two-domain (α - β) streptokinase isolated from *S. uberis* with specificity toward bovine plasminogen. The interaction between streptokinase and plasminogen occurred in two ways: first a rapid association of the proteins and second a slow transition to the active complex.

Despite the multiplicity of bacterial activities which must be employed by *S. uberis* in the colonization of the bovine mammary gland, the plasminogen activator is currently the only antigen to be assigned a putative role in this process. Vaccination with plasminogen activator, which induced a neutralizing antibody response, reduced the rate of colonization and decreased the incidence of the disease following experimental challenge (Leigh et al. 1999). A subunit vaccine, based on the plasminogen activator, produced by Leigh et al. (2000) showed cross-protective effects following experimental challenge.

In a more recent investigation Ward and Leigh (2002) identified a gene *PauB* encoding a plasminogen activator which was isolated from *pauA* negative *S. uberis* strains. *PauB* had a molecular weight of 30 kDa. However, these authors found a low ratio of appearance among *S. uberis*.

2.4.6 Lactoferrin-Binding Proteins

Lactoferrin (Lf), an iron-binding glycoprotein present in bovine milk, increases dramatically during involution and during infection of the mammary gland. Lf is one of the non-specific antibacterial systems in bovine milk (Smith and Oliver 1981). Bacterial iron deprivation has been considered the mechanism of Lf-mediated antibacterial action (Arnold et al. 1977). *S. uberis* and other streptococci are more resistant to the antibacterial effect of Lf than Gram-negative bacteria (Todhunter et al. 1985), probably because streptococci do not have a high requirement for iron (Weinberg 1978). In addition to binding iron, bovine Lf inhibited mitogen- and alloantigen-induced lymphocyte proliferation and decreased cellular proliferation of clonal bovine mammary epithelial cell line

MAC-T (Rejman et al. 1992a; Rejman et al. 1992b). Lactoferrin has been found to bind to several types of host cells, including human and bovine mammary epithelial cells (Rejman et al. 1994).

Fang and Oliver (1999) and Oliver et al. (2000) conducted a study on *S. uberis* for the identification of lactoferrin-binding proteins. The strains evaluated bound Lf in milk. By the use of polyacrylamide gel electrophoresis and western blotting it was observed that at least two proteins from *S. uberis* surface molecules were involved in Lf binding. Fang and Oliver (1999) determined the effects of Lf on adherence of *S. uberis* to mammary epithelial cells and phagocytic cells. The authors postulated Lf might function as a bridging molecule between bacteria and epithelial cells or phagocytic cells and might play a role in the pathogenesis of *S. uberis* mastitis. Similar findings were reported by Fang et al. (2000) who also observed an enhanced adherence of *S. uberis* strains to mammary epithelial cells by the addition of Lf to the culture medium. These studies also suggested that Lf could function as a bridging molecule between *S. uberis* and bovine epithelial cells, and might facilitated the adherence of the bacteria to the cells.

2.4.7 Amino Acid and Opine Binding Proteins

It has been demonstrated that a wide variety of substrates, such as sugars, peptides, anions and metals are imported by bacteria via binding protein dependent, ATP-binding cassette type (ABC-type) transport systems. In Gram-negative bacteria a periplasmic binding protein consists of two hydrophobic transmembrane proteins that form the translocation pathway and channel the substrate to the cytoplasm. The peripheral membrane ATP-binding proteins localized to the cytoplasmic site of the membrane are tightly associated with the transmembrane proteins and provide energy to the transport system by driving ATP hydrolysis (Higgins 1992; Fath and Kolter 1993).

Jiang et al. (1998) reported about the identification of a gene locus *abp* from *S. uberis*. This gene encoded a 31 kDa protein that, on the basis of sequence homology, is likely to be an amino acid binding protein (Abp) of an ABC-type amino acid transport system. In conclusion, an open reading frame capable of

coding a 277-residue polypeptide had been identified in *S. uberis* and this protein had sequence similarity to a number of polar amino acid and opine binding proteins of Gram-negative bacteria. The surface localization of Abp, the presence of a putative secretory signal sequence, and the consensus-cleavage site suggested that this protein could be a lipoprotein tied up to the cell membrane with an N-terminal glyceride-cysteine. On the basis of sequence homology and lipoprotein character it appears that the Abp belongs to a receptor family that transports polar amino acids and opines. This protein might serve as an attractive target for the development of new prophylactic compounds for the prevention of bovine mastitis. Thus, Abp appears to be a common protein present in *S. uberis* and absent in case of *S. parauberis*. This is in contrast to the CAMP factor which, although immediately downstream of *abp*, is present only in a limited number of strains (Jiang et al. 1996).

The presence of binding protein dependent transport systems in Gram-positive bacteria has only recently been documented. The oligopeptide transport system *amiA* of *Streptococcus pneumoniae* (Alloig et al. 1990) and *spoOK* and *app* of *Bacillus subtilis* (Perego et al. 1991; Rudner et al. 1991; Koide and Hoch 1994), the ribose and dipeptide transport systems *rbs* and *dciA* of *B. subtilis* (Woodson and Devine 1994; Mathiopoulos et al. 1991), the glutamine transport system of *Bacillus stearothermophilus* (Wu and Welker 1991), and the amino acid transport system of *B. subtilis* (Rodriguez and Grandi 1995) have been identified and characterized. It appeared that the overall structural organization of these systems is highly similar to their counterparts in Gram-negative bacteria. However, since Gram-positive organisms lack an outer membrane and consequently have no periplasm, the solute-specific binding proteins are lipoproteins with an N-terminal glyceride-cysteine that allows them to be tethered to the external surface of the cell membrane in the proximity of the integral cell membrane components of the transport system (Gilson et al. 1988; Tam and Saier 1993).

A nucleotide sequencing of gene locus *abp* from *S. uberis* was performed by Jiang et al. (1998). These studies revealed that the sequence of *abp* shared homologies with the polar amino acid and opine transport systems, particularly with the glutamine-binding protein and arginine-binding proteins of *E. coli* (Nohno

et al. 1986; Wissenbach et al. 1993), the histidine-binding protein and lysine-arginine-ornithine binding subunit of *Salmonella typhimurium* (Higgins and Ames 1981; Higgins et al. 1982), and the octopine-binding protein and the nonpaline-binding protein of *Agrobacterium tumefaciens* (Valdivia et al. 1991; Zanker et al. 1992). The observations showed that the *abp* locus of *S. uberis* encodes a protein homologous to polar amino acid and opine binding proteins of Gram-negative bacteria and concluded that this protein could have implications in the pathogenesis of bovine mastitis.

3. Materials and Methods

3.1 Collection of Milk Samples

For the present study 342 bovine milk samples from 342 quarters of 269 cows were collected from 93 different farms within three months from January to March 1999 at different locations in Hesse, Germany. The collection was performed with the kind cooperation of Dr. W. Wolter and Dr. M. Zschöck (Staatliches Medizinal-, Lebensmittel- und Veterinäruntersuchungsamt Mittelhessen, Giessen). Approximately 100 µl of the clinical (milk with clots) as well as subclinical (invisible abnormalities with high rise in total somatic cell count) samples were initially plated on blood agar while subclinical samples were subjected to total somatic cell count (SCC) in order to confirm the subclinical status of the collected samples. The determination of cell count was performed with the Fossomatic system (360 N. Foss Electric A/S, Hamburg, Germany) in cooperation with assistants of the Veterinäruntersuchungsamt.

3.2 Cultivation on Columbia Esculin Blood Agar

All previously collected isolates were subsequently cultivated on columbia esculin blood agar to determine the culturing ability and pattern of the isolates. The composition of the culturing media was as follows:

Columbia esculin agar base (Merck, Darmstadt, Germany)

Special peptone	23.0 g
Starch	1.0 g
Sodium chloride	5.0 g
Agar	13.0 g
Aqua dest	1000 ml
Esculin	0.5 g (additional ingredient to be added)

pH 7.3 ± 0.2 at 25°C

In accordance with the manufacturer's instruction the media was autoclaved (15 min at 121°C) and cooled at approximately 50°C. Finally 5%

aseptically collected, defibrinated sheep blood was gently mixed with the esculin agar base. The plates were poured and kept at 4°C for further use.

The isolates were cultivated and the plates incubated under aerobic conditions for 18-24 h at 37°C. The cultural pattern was later confirmed under UV light. The esculin positive strains showed no fluorescence under UV light.

3.3 Cultivation on Media Specific for Enterococci

For further differentiation the isolates were cultivated on five different selective media specific for the isolation of enterococci. An *Enterococcus faecalis* strain, obtained from the institute's strain collection, was used as a control. The media and their compositions were as follows:

3.3.1 Citrate Azide Tween Carbonate Agar [CATC Agar] (Merck)

The basic ingredients were as follows:

Peptone from pancreas	15.0 g
Yeast extract	5.0 g
Calcium hydrogen phosphate	5.0 g
Sodium citrate	15.0 g
Tween 80	1.0 g
Agar	15.0 g
Aqua dest	1000 ml

pH 7.0 ± 0.2 at 25°C

The media was prepared according to the prescribed instructions given by the manufacturer, autoclaved (15 min at 121°C), cooled at 50°C, then gently mixed with 20 ml of a 10% sodium carbonate solution, 10 ml of 1% 2, 3, 5-Triphenyl tetrazolium chloride and with 4 ml of a 10% sterile filtered solution of sodium azide per liter and poured for further use. The cultured isolates were incubated for 18-24 h at 37°C. The results were recorded on the basis of color and growth pattern of the isolates. Negative or weak growth indicated genus *Streptococcus*, red or reddish brown colonies genus *Enterococcus*.

3.3.2 Chromocult Enterococci Agar (Merck)

The composition of the media per liter was as follows:

Peptone	8.6 g
Sodium chloride	6.4 g
Sodium azide	0.6 g
Tween 80	2.2 g
5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside (X-Glu)	0.06 g
Agar	14.0 g
Aqua dest	1000 ml

pH 7.5 ± 0.2 at 25°C

Bacteria of genus *Streptococcus* showed no growth, enterococci grew with blue colonies.

3.3.3 Esculin Bile Agar (Oxoid, Wesel, Germany)

The composition of the media per liter was as follows:

Meat extract	3.0 g
Peptone from meat	5.0 g
Ox bile	40.0 g
Esculin	1.0 g
Iron (III) citrate	0.5 g
Agar	14.5 g
Aqua dest	1000 ml

pH 6.6 ± 0.2 at 25°C

According to the instructions of the manufacturer, the media was autoclaved at 121°C for 15 min, cooled up to $45\text{-}50^{\circ}\text{C}$ and mixed with 50 ml/l of bovine serum. Negative or weakly grown isolates indicated genus *Streptococcus*, whereas enterococci grew in black colonies.

3.3.4 Kanamycin Esculin Azide Agar [K.A.A] (Merck)

The composition of the media was as follows:

Peptone from casein	20.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Sodium citrate	1.0 g
Sodium azide	0.15 g
Kanamycin sulfate	0.02 g
Esculin	1.0 g
Ammonium iron (III) citrate	0.5 g
Agar	15.0 g
Aqua dest	1000 ml

pH 7.1 ± 0.2 at 25°C

Bacteria of genus *Streptococcus* showed no growth, enterococci grew with black colonies.

3.3.5 Slanetz-Bartley Media (Oxoid)

The composition of the media was as follows:

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dihydrogen phosphate	4.0 g
Sodium azide	0.4 g
2, 3, 5-triphenyl tetrazolium chloride	0.1 g
Agar	10.0 g
Aqua dest	1000 ml

pH 7.1 ± 0.2

According to manufacturer's instructions the media was poured after sterile filtration. The media was not subjected to autoclaving or boiling.

Streptococci showed no or weak growth whereas enterococci grew with red colonies.

After cultivation of the isolates, the growth patterns on the various media were recorded.

Finally, on the basis of the above mentioned cultural ability and growth patterns 131 isolates from 112 cows of 59 different farms effected with subclinical and clinical mastitis were further processed. The location of the farms in Hesse is shown in fig. 1.

In addition the two *S. uberis* reference strains NCDO 2038 and NCDO 2086 and the *S. parauberis* reference strain NCDO 2020, obtained from the institute's strain collection, as well as the *S. parauberis* strain 94/16, originally isolated from a diseased turbot, were included. The latter was kindly obtained from J. F. Fernández-Garayzábal (Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain) (Doménech et al. 1996). The isolates were finally subcultured on sheep blood agar for further confirmation and characterization.

3.3.6 Nutrient Agar Base (Merck)

Routine subculturing of the isolates was performed on nutrient blood agar. The composition of the media was as follows:

Heart extract	10.0 g
Tryptose	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Aqua dest	1000 ml

pH 6.8 ± 0.2 at 25°C

According to the manufacturer's instruction the media was autoclaved (15 min at 121°C) and cooled at approximately 50°C. Finally 5% aseptically collected, defibrinated sheep blood was gently mixed, the plates were poured and kept at 4°C for further use.



Figure 1. Distribution of 59 farms with code numbers of the farms from different locations in Hesse, Germany from where the isolates were collected.

3.3.7 Todd-Hewitt Broth [THB] (Oxoid)

A cultivation of the bacteria in fluid media was performed in Todd-Hewitt broth.

The composition of the media was as follows:

Casein	20.0 g
Flesh infusion from cattle	10.0 g
Sodium bicarbonate	2.0 g
Sodium chloride	2.0 g
Disodium hydrogen phosphate	0.4 g
Glucose	2.0 g
Aqua dest	1000 ml

pH 7.8 ± 0.2

An incubation of the bacteria was carried out for 18-24 h at 37°C under aerobic conditions with or without using a shaker.

3.3.8 Brain Heart Infusion [BHI] (Merck)

A cultivation of the bacteria in soft agar was performed in brain heart infusion.

The basic ingredients were as follows:

Brain, heart extract and peptone	27.5 g (Nutrient substrate)
Glucose	2.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	2.5 g
Aqua dest	1000 ml

pH 6.8 ± 0.2 at 25°C

The broth was mixed with 0.2% agar (Merck) and dispensed (10 ml) in glass tubes. Finally the glass tubes were subjected to sterilization in a moist chamber for 1 h at 95°C.

3.3.9 Mueller-Hinton Nutrient Media (Oxoid)

For the antibiotic sensitivity test a cultivation of the bacteria was performed on Mueller-Hinton agar.

The composition of the media was as follows:

Beef, dehydrated infusion from 300g	2.0 g
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Aqua dest	1000 ml

PH 7.4 ± 0.2 at 25°C

The media was autoclaved (15 min at 121°C) and cooled at approximately 50°C . Finally 5% aseptically collected, defibrinated sheep blood was gently mixed. The plates were poured and kept at 4°C for further use.

3.4 Identification

A further identification of the bacteria was performed as described by Facklam and Carey (1985), Schleifer (1989), Lämmler (1991), Lämmler and Hahn (1994).

3.4.1 Biochemical Characterization

3.4.1.1 Carbohydrate Fermentation Tests

For this purpose 5 ml phenol-red broth (Merck) served as basic medium. The carbohydrates arabinose, fructose, inulin, lactose, maltose, mannitol, raffinose, ribose, saccharose, salicin, sorbitol and trehalose (Merck) were added to a final concentration of 1%. All the prepared carbohydrates were sterilized by autoclaving for 6 min at 121°C . After incubation for 24-48 h at 37°C positive reactions exhibited a distinctively yellow color from previously red media.

3.4.1.2 Arginine Hydrolysis (ADH)

The test was conducted as described by Casals and Pringler (1993). A dense “milky suspension” (at least McFarland No. 2) from the isolate to be tested was prepared in 0.25 ml sterilized 0.14 mol/l sodium chloride in a narrow glass tube. One arginine dihydrolase (ADH) diagnostic tablet (Rosco, Hiss Diagnostics, Freiburg, Germany) and 3 drops of sterile paraffin oil were added. After closing the tube and an incubation for 4 h or up to 18-24 h at 37°C, a formation of a red color was recorded as positive reaction and a yellow or an orange color as negative reaction.

3.4.1.3 Esculin Hydrolysis

For confirmation of esculin hydrolysis 5 ml sterile BHI (3.3.8) was used as nutrient media. To this 0.1% esculin and 0.05% iron (III) citrate was added and autoclaved. Two drops of bacterial suspension were added to the tubes and subjected to an overnight incubation at 37°C. A hydrolysis of esculin exhibited a black coloration of the media.

3.4.1.4 Hippurate Hydrolysis

The test was carried out on the basis of the method described by Hwang and Ederer (1975). For this the isolates were suspended in 0.4 ml of a 1% sodium hippurate solution (Merck), incubated for 2 h at 37°C followed by an addition of 0.2 ml of a 3.5% ninhydrin solution (Merck). This was incubated for 10 min at 37°C. A blue-violet color of the media was recorded as a positive reaction.

3.4.2 Determination of Enzyme Activities

3.4.2.1 β -D-Glucuronidase

The test was carried out according to the information given by Kilian and Bülow (1976). A dense “milky suspension” (at least McFarland No. 2) from

the strain to be tested was prepared in 0.25 ml sterilized 0.14 mol/l sodium chloride in a narrow glass tube. One β -D-glucuronidase diagnostic test tablet (Rosco, Hiss Diagnostics) was added, the tube closed and incubated for 4 h or up to 18-24 h at 37°C. A formation of a yellow color was recorded as positive reaction, a colorless reaction was recorded as negative.

3.4.2.2 Pyrrolidonyl Aminopeptidase

The test was performed according to Wellstood (1987). For this a dense “milky suspension” (at least McFarland No. 2) was prepared from the isolates to be tested in 0.25 ml sterilized 0.14 mol/l sodium chloride in a narrow glass tube. Subsequently a diagnostic test tablet (Rosco, Hiss Diagnostics) was added, the tube tightly plugged and incubated for 4 h or up to 18-24 h at 37°C. After incubation and the addition of 3 drops of aminopeptidase reagent (Rosco, Hiss Diagnostics) a color reaction could be recorded within 5 min. The formation of a red color indicated a positive reaction, a yellow/orange color a negative reaction.

3.4.2.3 Hyaluronidase

The plate test for the detection of hyaluronidase positive isolates was performed according to Winkle (1979). For this the isolates were cultivated in close proximity of the mucoid growing *S. equi* subsp. *zooepidemicus* strain W60. The *S. equi* subsp. *zooepidemicus* strain was obtained from the institute’s strain collection. A growth of the indicator strain in non mucoid colonies in close proximity of the isolate to be tested indicated a positive reaction.

3.4.3 Serogrouping

3.4.3.1 Antigen Extraction

An antigen extraction was performed using the autoclave extraction method described by Rantz and Randall (1955). For this the isolates were

cultivated in 40 ml THB (3.3.7) for 18-24 h at 37°C. The bacterial suspension was centrifuged (10 min. 10000xg, 4°C, Sorvall type RC-2B, Newton, Connecticut, USA), the supernatant decanted and the bacterial pellet washed in 5 ml of a sterilized 0.14 mol/l sodium chloride solution. The centrifugation process was repeated. The supernatant was discarded and the pellet resuspended in 0.5 ml of a 0.14 mol/l sodium chloride solution. The bacterial suspension was neutralized by the addition of 1 mol/l sodium hydroxide with 0.05% alcoholic phenol red serving as indicator. The suspension was autoclaved for 20 min at 120°C and centrifuged. The supernatant kept at 4°C for further use was used for the detection of group antigen.

3.4.3.2 Group Specific Antisera

Specific antisera formerly prepared against group antigen of serogroup A, B, C, D, E, L, P, U and V reference strains were kindly provided by Prof. Dr. Ch. Lämmler (Milchwissenschaften, Institut für Tierärztliche Nahrungsmittelkunde, Justus-Liebig-Universität, Giessen).

3.4.3.3 Double Immunodiffusion Test

For the serological identification of polysaccharide antigens, 0.4 g agarose (Standard EEO, Serva, Heidelberg, Germany), 1.2 g polyethylene glycol (PEG Type 6000, Serva) and 0.03 g sodium azide (Merck) in 20 ml aqua dest and 20 ml (0.5 mol/l, pH 7.5) phosphate buffered solution (PBS) were mixed and boiled till the agarose and PEG were completely homogenized. Of this agarose solution 20 ml were carefully poured with a sterile glass pipette on a rail containing six well fixed clean glass slides. After solidification of the gel one central and four marginal perforations were laid out with the help of a gel punch (LKB, Stockholm, Sweden). The distance between the holes measured about 2.5 mm. Of the antigen extracts (3.4.3.1) 5 µl were dispensed in the marginal hole and 5 µl of group specific antisera in the central hole. The diffusion reaction could successfully be recorded after 18-24 h incubation at room temperature (25°C) in a moist chamber.

3.4.4 Lectin Agglutination Test

For this the bacteria were cultivated in 5 ml THB (3.3.7) for 24 h at 37°C and subsequently subjected to centrifugation (10 min, 10000xg, 4°C). The supernatant was discarded, the pellet washed in 5 ml PBS (3.4.3.3), centrifuged and finally resuspended in 200 µl PBS. For the inhibition of self agglutination 5 µl trypsin (1 mg trypsin/ml PBS) was added to the bacterial suspension and incubated for one h at 37°C. This bacterial suspension was used for further lectin agglutination reactions. The lectin extracts of *Dolichos biflorus* (Sigma, Deisenhofen, Germany) were used in a concentration of 0.25 mg/ml PBS, and *Helix pomatia* (Sigma) in a concentration of 20 µg/ml PBS. On two sides of a clean glass slide 20 µl of bacterial suspensions were dispensed and mixed with 20 µl of each lectin, respectively, for one min. Agglutination reactions could be observed using an illuminator. A mixture of PBS and 20 µl of the bacterial suspension served as control.

3.5 Phenotypic Characterization

3.5.1 Determination of CAMP Reaction

For this purpose a β-hemolytic *Staphylococcus aureus* culture (reference strain Pertsch) was inoculated vertically and the test strain horizontally up to 3-5 mm to the staphylococcal inoculation line (Lämmle and Hahn 1994). A positive reaction could be observed after incubation for 18-24 h at 37°C as a half moon forming zone of complete hemolysis in the zone of incomplete staphylococcal-β-hemolysis. The *Streptococcus agalactiae* strain COH I was used as a positive control.

3.5.2 Growth Pattern in Fluid Media

The determination of the growth pattern in fluid media was performed as described by Wibawan and Lämmle (1991). The bacterial strains to be tested were cultivated in 10 ml THB (3.3.7) for 18-24 h at 37°C without agitating the glass

tubes. The bacterial growth pattern was recorded as turbid (uniform turbidity of whole broth) or clear (sediment formation with a clear supernatant).

3.5.3 Growth Pattern in Soft Agar

The investigation of the growth pattern of the bacteria in soft agar was performed as described by Yoshida (1971). For this purpose 0.1 ml of the cultured bacteria (3.5.2) was diluted 1:1000 in sterilized 0.14 mol/l sodium chloride. Of this 0.1 ml was inoculated in 10 ml BHI (3.3.8) at approximately 40°C, vortexed and incubated for 18-24 h at 37°C. The colony morphology were recorded as compact or diffuse according to Yoshida (1971) and Wibawan and Lämmle (1991).

3.5.4 Salt Aggregation Test

The salt aggregation test was carried out according to Johnson and Wadström (1984). For this the bacterial strains were cultured in 10 ml THB (3.3.7) and incubated for 18-24 h at 37°C. The cultured broth was subjected to centrifugation (10 min x 3000xg) and washed 2x each time with 0.001 mol/l sodium phosphate (Na_2PO_4) buffer (pH 6.8). This was followed by a photometric adjustment (Spectronic 20, Bausch and Lomb, New York, USA) of the bacteria at 620 nm and 10% transmission corresponding to a bacterial number of approximately 10^9 /ml. After gentle mixing of 10 μl of the prepared bacterial suspension with 10 μl of different concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 1.8, 2.0, 2.4, 3.0 and 3.4 mol/l ammonium sulfate [$(\text{NH}_4)_2\text{SO}_4$], respectively, on a clean glass slide for 30 sec a positive bacterial aggregation could be observed under indirect light. For control purposes the bacterial suspension was mixed with 10 μl sodium phosphate.

3.5.5 Hemagglutination Reaction

3.5.5.1 Collection of Erythrocytes

The preparation of erythrocyte suspension was performed according to the method described by Wibawan et al. (1993), using 9 ml of rabbit blood mixed

with 1 ml sterile sodium citrate (0.2 mol/l, pH 5.2) as anticoagulant. This was centrifuged (10 min, 2000xg), the supernatant collected in a separate sterile tube and the sediment washed twice with sterilized 0.14 mol/l sodium chloride. After centrifugation the pellet was used to prepare a 2% erythrocyte suspension.

3.5.5.2 Slide Hemagglutination Test

The slide test was carried out according to Wibawan et al. (1993). A hemagglutination positive (395/2) as well as a hemagglutination negative (G28) group B streptococcal strain served as control (Wibawan et al. 1993). The bacteria were cultured in 10 ml THB (3.3.7) for 18-24 h at 37°C and centrifuged (10 min, 3000xg). The supernatant was discarded and the pellet washed twice with 0.002 mol/l buffered phosphate (0.36 g/l Na₂HPO₄ x 2H₂O, pH 6.8). The bacteria were adjusted photometrically to 10% transmission (10⁹ bacteria/ml) at 620nm with the help of a spectrophotometer (3.5.4). Approximately 25 µl of the prepared bacterial suspension was dispensed on a clean glass slide and mixed with 25 µl of the prepared erythrocyte suspension. After approximately 30 sec a positive reaction could be seen as clearly visible agglutination. Bacterial and erythrocyte suspensions (25 µl) mixed with 25 µl 0.002 mol/l buffered phosphate (pH 6.8), respectively, served as control.

3.5.6 Determination of Antibiotic Sensitivity Test

The antibiotic sensitivity tests were performed according to the recommendations of the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV, Berlin) (Stand VIII 92). Using these instructions 4-5 identical colonies of strains to be tested were incubated in 3 ml THB (3.3.7) for 2 h at 37°C. After incubation 0.1 ml of the bacterial suspension was plated on Mueller-Hinton nutrient agar (3.3.9) with the help of a sterile glass spreader. The following antibiotic test disks (Becton Dickinson, Heidelberg, Germany) were laid on the dried inoculum with the help of a dispenser.

Cefacetril	30 µg	
Cefotaxim	30 µg	
Clindamycin	2 µg	
Colistin	10 µg	
Erythromycin	15 µg	
Gentamicin	10 µg	
Minocyclin	30 µg	
Penicillin-G	10 IU	
Sulfamethaxazole/Trimethoprim (SXT)	23.75/1.25 µg	
Tetracycline	30 µg	

The plates were kept at room temperature for 30 minutes and then subjected to incubation for 18-24 h at 37°C. The zone of inhibition was estimated and compared according to data of BgVV, (Stand VIII 92) as well as Barry and Thornsberry (1991).

3.6 Molecular Characterization by Polymerase Chain Reaction (PCR)

3.6.1 Extraction and Preparation of Bacterial DNA

The extraction and preparation of DNA as 'template' for PCR reaction was performed as described by Bentley et al. (1993). For this purpose few colonies of the bacteria were taken from blood agar media (3.3.6) and suspended in 100 µl TE-buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0), containing 4 µl mutanolysin (10 U/µl; Sigma). This was incubated in a water bath for one h at 37°C. For deproteinization 10 µl proteinase K (14.8 mg/ml; Boehringer, Mannheim, Germany) was added and further incubated for 2 h at 56°C in a water bath. The sample was boiled for 10 min at 100°C to inactivate proteinase K action, followed by a centrifugation (5 sec, 10000xg). The supernatant was cooled and used as template in PCR.

3.6.2 Amplification of the 16S rRNA Gene by PCR

A PCR targeted to the gene encoding the 16S rRNA was performed by use of the oligonucleotide primer ARI described by Bentley and Leigh (1995). The sequence of the oligonucleotide primer AmII used as primer 2 was described by Abdulmawjood and Lämmler (1999). The oligonucleotide primers were synthesized by MWG-Biotech (Ebersberg, Germany) with the sequences 5' primer 1 ARI 5'-GAG AGT TTG ATC CTG GCT CAG GA- 3' and primer 2 AMII 5'-CGG GTG TTA CAA ACT CTC GTG GT- 3'. The PCR reaction mixture was initially carried out by the preparation of a master mix.

The master mix for each assay contained:

Aqua bidest	20.0 µl
Incubation buffer	3.0 µl (10x buffer; Promega, Mannheim, Germany)
MgCl ₂	1.8 µl (25 mmol/l; Promega)
dNTP	0.6 µl (10 mmol/l; MBI Fermentas, St. Leon-Rot, Germany)
Primer 1	1.0 µl (10 pmol/l)
Primer 2	1.0 µl (10 pmol/l)
Taq-DNA Polymerase	0.1 µl (5 U/µl; Promega)

Finally 27.5 µl of the master mix was dispensed in 0.2 ml sterile PCR reaction tubes and mixed with 2.5 µl of the prepared DNA (3.6.1). The reaction tubes were subjected to 30 cycles in a thermal cycler (Techne-Progene, Thermodux, Wertheim, Germany) with the following program: 1x4 minutes precycle at 94°C, 30 cycles of denaturation at 94°C for 1.5 min, primer annealing at 56°C for 1.5 min and extension at 72°C for 1.5 min. The completion of the final cycle was followed by a final extension incubation of 72°C for 5 min. The amplicons were kept at 4°C for further use.

3.6.3 Restriction Analysis of 16S rRNA Gene Products

3.6.3.1 Enzymatic Digestion of Amplified DNA

Restriction fragment analysis of the amplified 16S rRNA gene (3.6.2) was performed as recommended by Jayarao et al. (1991). Amplified DNA was

digested for one h at 37°C in a water bath in 30 µl volumes with *RsaI*, *AvaII* and *MspI* (New England Biolabs, Frankfurt(M), Germany) restriction enzymes, respectively. The restriction endonuclease reaction mixtures were prepared according to the following protocols.

***RsaI* restriction enzyme**

PCR product	18.0 µl
Enzyme	1.0 µl
Buffer (10x; Biolabs)	3.0 µl
BSA (10x; Biolabs)	3.0 µl
Distilled water	5.0 µl

***MspI* restriction enzyme**

PCR product	22.0 µl
Enzyme	2.0 µl
Buffer (10x; Biolabs)	3.0 µl
Distilled water	3.0 µl

***AvaII* restriction enzyme**

PCR product	22.0 µl
Enzyme	1.0 µl
Buffer (10x; Biolabs)	3.0 µl
Distilled water	4.0 µl

3.6.4 Agarose Gel Electrophoresis

For the investigation and confirmation of the restriction endonuclease digested 16S rRNA gene products the fragments were electrophoresed in a 2% agarose gel (Gibco) by using 1xTAE buffer (40 mmol/l Tris-HCl, 1 mmol/l EDTA, 1.14 mol/l glacial acetic acid) as running buffer. For this purpose 10 µl of the PCR product (3.6.3) was mixed with 3 µl 6x loading solution (MBI Fermentas) and loaded in the gel. The electrophoresis was carried out at 100 mA for four hours.

3.6.5 Ethidium Bromide Staining and Documentation

After successful completion of electrophoresis the gel was stained for five minutes with an ethidium bromide solution (5 µg/ml; Sigma). Ethidium bromide fluorescent staining material has the ability to interact and impregnate with the double stranded DNA. In order to remove the excessive staining material, the gel was rinsed in aqua dest for 20 minutes at room temperature. Further on the gel was carefully removed and the bands visualized by UV transilluminator and photographed (Pharmacia Biotech Image Master® VDS, Amersham Pharmacia Biotech, Freiburg, Germany). The fragments could be seen as bright bands in front of a dark background.

3.6.6 PCR Amplification of Species-Specific Parts of the Gene Encoding the 16S rRNA

The amplification of species-specific parts of the 16S rRNA gene, also including the V2-region of *S. uberis* and *S. parauberis*, was conducted on the basis of sequence data described by Brown and Brown (1995) (accession no. U41048) and Doménech et al. (1996) (accession no. X89967), respectively. The primer pairs for both species were used as described by Hassan et al. (2001). Primer sequences for *S. uberis* were ub-I 5'-CGC ATG ACA ATA GGG TAC A- 3' and ub-II 5'-GCC TTT AAC TTC AGA CTT ATC A- 3' and for *S. parauberis* paraub-I 5'-CAT GAC AAT TAA GTA CTC ATG TAC TA- 3' and paraub-II 5'-CAC CAC CTG TCA CCT CTG TC- 3'. The primers were synthesized by MWG-Biotech. The PCR temperature profile consisted of 30 cycles with the following program: 94°C for 60 sec (denaturation), 58°C for 1.5 min (primer annealing), 72°C for 1.5 min (extension), with a final extension step of 72°C for 5 min.

3.6.7 PCR Amplification of Species-Specific Parts of the 16S-23S rDNA Intergenic Spacer Region

For the amplification of *S. uberis* specific regions of the 16S-23S rDNA intergenic spacer the oligonucleotide primer 1 STRAU-ub-I 5'-TAA GGA ACA

CGT TGG TTA AG- 3' and primer 2 STRAU-ub-II 5'-TCC AGT CCT TAG ACC TTC T- 3' were used as recommended by Forsman et al. (1997). The PCR program consisting of 30 cycles was carried out as follows: 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec. The final cycle was followed by an extension incubation of 72°C for 5 min. A primer design for the amplification of species-specific parts of 16S-23S rDNA intergenic spacer region of *S. parauberis* was performed by Hassan et al. (2001). Primer 1 paraub-16S-23S-I had the sequence 5'-AAA TGG AAG CAC GTT AGG AAA- 3' and primer 2 paraub-16S-23S-II the sequence 5'-GCA AGC CGA ACA TCT CTT TG- 3'. The PCR was performed with the following program consisting of 30 cycles: 94°C for 10 sec, 58°C for 10 sec, 72°C for 10 sec. The final cycle was followed by an extension incubation at 72°C for 5 min.

3.6.8 PCR Amplification of Species-Specific Parts of the Gene Encoding the 23S rRNA

The amplification of the 23S rRNA gene of the *S. uberis* and *S. parauberis* strains were carried with sequence data given by Harland et al. (1993). These sequences were obtained from data bank accession numbers X68038 and X68036, respectively. The primer sequences for *S. uberis* were ub-23S-I 5'-CGT ATT TAA AAT TGA CTT TAG CC- 3' and ub-23S-II 5'-AAT TTC TCC GCT ACC CAC- 3' and for *S. parauberis* paraub-23S-I 5'- AAA ATA GTA AAT GAC TCT AGC AGT- 3' and paraub-23S-II 5'-CGG AGA GAA CCA GCT ATC- 3' (Hassan et al. 2001). The PCR was performed with the following temperature program consisting of 30 cycles: 94°C for 45 sec, 64°C for 45 sec, 72°C for 45 sec, with a final extension step of 72°C for 5 min.

3.6.9 PCR Amplification of CAMP Factor Gene *cfu*

The gene *cfu* encoding the CAMP factor of *S. uberis* was described by Jiang et al. (1996) (accession no. U34322). For amplification of *S. uberis* CAMP factor gene *cfu* the oligonucleotide primer pairs with the sequence CAMP ub-I 5'-TTA ACA AGT GTC CAA GCA AAT CA- 3' and CAMP ub-II 5'-TTA GAA GGG AAT CGA CAT ACA AGT- 3', recommended by Hassan et al. (2000), were

used. The PCR temperature profile consisted of 30 cycles with the following program: 92°C for 60 sec, 58°C for 1.5 min, 72°C for 1.5 min, with a final extension step of 72°C for 5 min.

In parallel, a second CAMP factor gene specific primer pair was designed by using computer program OLIGO 4. The primer 1 had the sequence *cfu-I* 5'-CTT TAT TTT CCC CAA- 3' and primer 2 the sequence *cfu-II* 5'-ATT TCT TGG TCA ACT TGT- 3'. The PCR temperature program of 30 cycles was: 92°C for 60 sec, 45°C for 1.5 min, 72°C for 1.5 min. The final cycle was followed by an extension at 72°C for 5 min.

3.5.10 PCR Amplification of *S. uberis* Streptokinase/Plasminogen Activator Gene *skc/pauA*

The amplification of the *skc* gene of *S. uberis* was done by using the oligonucleotide primers SKC-I as primer 1 with the sequence 5'-CTC CTC TCC AAC AAA GAG G- 3' and SKC-II as primer 2 with the sequence 5'-GAA GGC CTT CCC CTT TGA AA- 3'. The primer sequence for *skc* was obtained from Rosey et al. (1999) (accession no. 012549). The PCR temperature program consisted of 30 cycles: 94°C for 60 sec, 52°C for 60 sec and 72°C for 90 sec. The sequence used for *pauA* amplification was for primer 1 P38 5'-AAT AAC CGG TTA TGA TTC CGA CTA C- 3' and for primer 2 P39 5'-AAA ATT TAC TCG AGA CTT CCT TTA AGG- 3'. The primer sequence was described by Johnsen et al. (1999) (accession no. AJ131604). The thermal cycler program consisted of 30 cycles: 94°C for 60 sec, 54°C for 60 sec and 72°C for 90 sec. The final cycle was followed by an extension incubation at 72°C for 5 min, respectively.

3.7 Macrorestriction Analysis of Chromosomal DNA

The DNA-fingerprinting was performed by preparation of whole bacterial DNA in agarose gel blocks and subsequent digestion of the bacterial DNA with restriction enzymes. The fragment pattern was finally studied by means of pulsed-field gel electrophoresis (PFGE).

3.7.1 DNA Preparation

The preparation and digestion of the genomic DNA was a modification of the procedure described by Maslow et al. (1993) as well as Thiele et al. (1993). A 40 ml flask of THB (3.3.7) was inoculated with a single colony of a 24 to 48 h old culture of the bacterial sample grown on sheep blood agar (3.3.6). The inoculated broth was incubated aerobically for 18-24 h at 37°C. The flask was rotated gently to suspend the cellular debris at the bottom of the flask into solution. The broth culture was centrifuged at 10000xg for 10 min at 4°C. The supernatant was discarded and the pellet washed, resuspended in 250 µl TE buffer (3.6.1) and photometrically adjusted to 5% transmission at 620 nm by using a spectrophotometer (3.5.4). Of the cell suspension 200 µl was mixed with an equal volume of 1% low melting and low gelling Incert agarose (Biozyme Diagnostic, Hessisch Oldenburg, Germany) at 55°C, carefully dispensed into 100 µl plug moulds and left to solidify on an ice block for at least 10 min. After solidification the gel block was placed in 200 µl lysis buffer [6 mmol/l Tris, pH 7.6, 1 mol/l NaCl, 10 mmol/l EDTA, pH 7.6, 0.5% Brij-58 (Sigma), 0.2% sodium dodecyl sulfate (Sigma), 0.5% sodium lauroyl sarcosin (Sigma)] supplemented with 1 mg/ml lysozyme (Merck) and incubated overnight in a water bath at 37°C. Later a deproteinization was carried out by the addition of proteinase K (final concentration 0.5 µg/ml; Boehringer) followed by an overnight incubation at 56°C in a water bath. The lysis buffer was decanted and the blocks washed twice (30 min in 200 µl TE buffer/block) at room temperature. An inactivation of proteinase K was performed by the addition of phenylmethylsulfonylfluoride (PMSF; Sigma), incubated for one h at 56°C in a water bath, with a final concentration of 1.0 mmol/l. The latter was repeated one time. After two times washing of each block with 200 µl TE buffer each block was incubated at 4°C in TE buffer for further use.

3.7.2 DNA Digestion

After preparation, the DNA was digested with the following restriction enzymes.

1. Digestion with *Sma*I restriction enzyme

DNA (per block)

Buffer A (10x; Biolabs) 20.0 μ l

*Sma*I (25U; Biolabs) 2.0 μ l

Aqua dest 178.0 μ l

According to the manufacturer's instructions, each block was incubated for 8 h at 25°C.

2. Digestion with *Apa*I restriction enzyme

DNA (per block)

Buffer 3 (10x; Boehringer) 20.0 μ l

*Apa*I (40U; Boehringer) 2.0 μ l

Aqua dest 178.0 μ l

According to the manufacturer's instructions, each block was incubated for 25 h at 30°C.

3.7.3 Pulsed-Field Gel Electrophoresis

The fragments of the digested genomic DNA (3.7.2) were loaded into wells of a 1% agarose gel (Molecular Biology Certified Agarose, Bio-Rad, Munich, Germany) in 0.5xTBE buffer (Tris-Borate buffer; 45 mol/l Tris, 45 mol/l Borate, 1.0 mol/l EDTA, pH 8.0), containing 0.5 μ g/ml ethidium bromide (Sigma). The wells were sealed with 1% agarose gel, (Agarose MP; Boehringer) without addition of ethidium bromide. The DNA fragments were separated using the pulsed-field gel electrophoresis system CHEF-DRII (Bio-Rad) with 2 liters of 0.5xTBE as running buffer. The pulse times for both restriction enzymes were used as mentioned below:

1. *SmaI* (Baseggio et al. 2000)

Voltage 6 Volts/cm

Pulse time 1-20 sec (linear gradient)

Time duration 20 h

Temperature 14°C

2. *ApaI* (Fink 2002)

Voltage 6 Volts/cm

Pulse time 3.4-12.9 sec (linear gradient)

Time duration 25 h

Temperature 14°C

With each assay low range PFG markers (0.1-200kb; Biolabs) and lambda ladder PFG markers (50-1000kb; Biolabs) were included as molecular size standards.

On completion of the assay, the gel was illuminated with UV light and photographed (3.6.5).

The interpretation of the restriction pattern was performed by dendrogram analysis with the help of computer program GelCompar 4.0 (Applied Maths BVBA, Kortrijk, Belgium).

4. Results

4.1 Identification and Phenotypic Characterization

The 337 collected milk samples of 337 quarters of 265 cows with subclinical mastitis (five samples of five quarters of four cows were obtained from clinical mastitis) were subjected to somatic cell count (SCC) by an electric cell counter indicating the subclinical status of mastitis. Among the 342 investigated samples 131 tested positive for *S. uberis* and *S. parauberis*.

All 131 streptococci isolated from the milk samples and the two *S. uberis* and the two *S. parauberis* reference strains could successfully be cultivated on sheep blood agar. Under microscopic examination the bacteria were Gram-positive cocci forming chains.

On the basis of hemolytic characteristics 126 isolates exhibited an α -hemolysis, the remaining nine strains were non-hemolytic. In addition four of the 131 streptococci grew with mucoid colonies.

According to the following results 130 of the 131 streptococci investigated could be identified as *S. uberis*, one isolate as *S. parauberis*.

All 131 isolates degraded esculin after cultivation on Columbia esculin blood agar. None of the 131 isolates grew on Citrate azide tween carbonate agar (CATC) (3.3.1) and Kanamycin esculin azide agar [K.A.A] (3.3.4). However, seven (5%), nine (7%) and four (3%) of the 131 isolates grew on Chromocult enterococci agar (3.3.2), Esculin bile agar (3.3.3) and Slanetz-Bartley agar (3.3.5), respectively. The reference strains of both species could not be cultivated on all five media specific for enterococci (Table 1).

Table 1. Cultivation and growth of the 131 isolates and the *S. uberis* and *S. parauberis* reference strains on different growth media

n	Columbia esculin blood agar	CATC agar	Chromocult enterococci agar	Esculin bile agar	K.A.A	Slanetz-Bartley agar
131	131* (100%)	0 (0%)	7** (5%)	9** (7%)	0 (0%)	4** (3%)
<i>S. uberis</i> NCDO 2038, NCDO 2086 <i>S. parauberis</i> NCDO 2020, 94/16	4* (100%)	0 (0%)	0 (0%)	0 (0%)	(0%)	0 (0%)

n = Number of strains

* = Number of strains showing growth (%)

** = Weak growth

The biochemical characteristics of the 131 isolates and the four reference strains are summarized in table 2. All isolates exhibited degradation of fructose, glucose, maltose, mannitol, saccharose, salicin, sorbitol and trehalose and hydrolyzed esculin and sodium hippurate. All except one of the isolates fermented arginine, lactose and ribose while all isolates did not ferment arabinose. Among the 131 streptococci investigated 90 (69.2%) and two (1.5%) strains fermented inulin and raffinose, respectively.

Table 2. Biochemical properties of *S. uberis* and *S. parauberis*

	<i>S. uberis</i> n = 130	<i>S. parauberis</i> n = 1	<i>S. uberis</i> NCDO 2038, NCDO 2086 n = 2	<i>S. parauberis</i> NCDO 2020, 94/16 n = 2
Arabinose	0* (0%)	0 (0%)	0 (0%)	0 (0%)
Arginine	129 (99%)	1 (100%)	2 (100%)	2 (100%)
Esculin	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Fructose	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Glucose	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Inulin	90 (69%)	0 (0%)	2 (100%)	2 (100%)
Lactose	129 (99%)	1 (100%)	2 (100%)	2 (100%)
Maltose	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Mannitol	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Na-Hippurate	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Raffinose	2 (1.5%)	0 (0%)	0 (0%)	2 (100%)
Ribose	129 (99%)	1 (100%)	2 (100%)	2 (100%)
Saccharose	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Salicin	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Sorbitol	130 (100%)	1 (100%)	2 (100%)	2 (100%)
Trehalose	130 (100%)	1 (100%)	2 (100%)	2 (100%)

n = Number of strains

* = Number of positive strains (%)

Further biochemical characteristics of the 131 streptococci and the reference strains investigated are shown in table 3. Among the 131 strains studied all *S. uberis* and the *S. uberis* reference strains NCDO 2038 and NCDO 2086 were β -D-glucuronidase positive. The *S. parauberis* strain and the *S. parauberis* reference strains NCDO 2020 and 94/16 were β -D-glucuronidase negative. Investigating pyrrolidonyl aminopeptidase enzyme activities 116 isolates and the four reference strains exhibited a positive reaction.

Investigating hyaluronidase enzyme activity 47 (35%) of the isolates produced the enzyme hyaluronidase indicated by the development of non-mucoid colonies of the mucoid growing *S. equi* subsp. *zooepidemicus* indicator strain. The remaining strains, also including the reference strains, appeared to be hyaluronidase negative.

Table 3. Enzyme activities of *S. uberis* and *S. parauberis*

	<i>S. uberis</i> n = 130	<i>S. parauberis</i> n = 1	<i>S. uberis</i> NCDO 2038, NCDO 2086	<i>S. parauberis</i> NCDO 2020, 94/16
β -D-glucuronidase	130*(100%)	0 (0%)	2 (100%)	0 (0%)
Pyrrolidonyl aminopeptidase	115 (88%)	1 (100%)	2 (100%)	2 (100%)
Hyaluronidase	47 (35%)	0 (0%)	0 (0%)	0 (0%)

n = Number of strains

* = Number of positive strains (%)

Serogrouping performed by double immunodiffusion tests revealed positive reactions for the investigated *S. uberis* and *S. parauberis* strains with Lancefield's group E, P, U, G and A specific antisera as shown in fig. 2 and 3. Among the *S. uberis* isolates 42 (32%) strains developed a reaction with group E specific antiserum and 11 (8%) and nine (7%) strains with group P and group U specific antiserum, respectively. One of the *S. uberis* strains reacted with antiserum A, whereas two strains simultaneously reacted with both antisera E and P as well as E

and U, respectively. Among the four *S. uberis* and *S. parauberis* reference strains two (50%) strains exhibited a positive reaction with group E specific antiserum and one strain with group G specific antiserum. The remaining 66 (49%) *S. uberis*, one strain of *S. parauberis* and one reference strain of *S. parauberis* failed to show a reaction with any of the antisera used and were categorized as non-groupable. The results are summarized in table 4.

Table 4. Serogrouping of *S. uberis* and *S. parauberis* strains with serogroup A, B, C, E, G, L, P, U, and V specific antisera

Serogroups	<i>S. uberis</i> n = 130	<i>S. parauberis</i> n = 1	<i>S. uberis</i> NCDO 2038, NCDO 2086 n = 2	<i>S. parauberis</i> NCDO 2020, 94/16 n = 2
A	1* (1%)	0 (0%)	0 (0%)	0 (0%)
B	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C	0 (0%)	0 (0%)	0 (0%)	0 (0%)
E	42 (32%)	0 (0%)	1 (50%)	1 (50%)
G	0 (0%)	0 (0%)	1 (50%)	0 (0%)
L	0 (0%)	0 (0%)	0 (0%)	0 (0%)
P	11 (8%)	0 (0%)	0 (0%)	0 (0%)
U	9 (7%)	0 (0%)	0 (0%)	0 (0%)
V	0 (0%)	0 (0%)	0 (0%)	0 (0%)
E and P	1 (1%)	0 (0%)	0 (0%)	0 (0%)
E and U	1 (1%)	0 (0%)	0 (0%)	0 (0%)
Non-groupable	66 (50%)	1 (100%)	0 (0%)	1 (50%)

n = Number of strains

* = Number of positive strains (%)

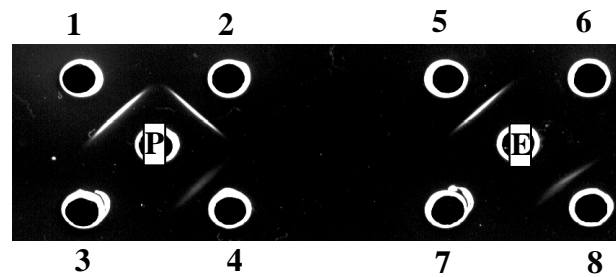


Figure 2. Double immunodiffusion reactions of serogroup P and E specific antisera with autoclave extracts of *S. uberis* (1, 2) and *S. parauberis* (5). Control strains of serogroup P and E (4, 8). Non-groupable strains are shown in well 3, 6 and 7.

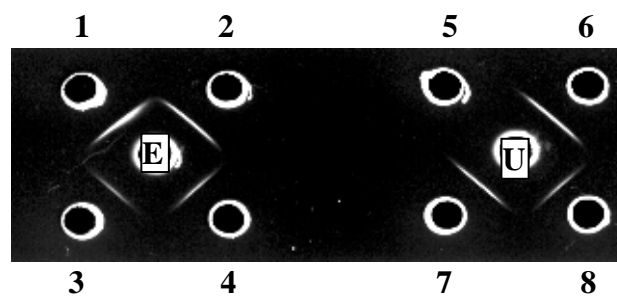


Figure 3. Double immunodiffusion reactions of serogroup E and U specific antisera with autoclave extracts of *S. uberis* strains (1, 2, 3, 5 and 6). Control strains of serogroup E and U (4, 8). A non-groupable strain is shown in well 7.

All 130 *S. uberis* strains, the one *S. parauberis* strain and the four *S. uberis* and *S. parauberis* reference strains were additionally examined for lectin agglutination reactions with the lectins from *Helix pomatia* and *Dolichos biflorus*. Among the *S. uberis* strains 43 (32.5%) exhibited agglutination reactions with the lectin of *Helix pomatia*. None of the remaining *S. uberis* and *S. parauberis*, also including the reference strains, showed a comparable reaction with any of the lectins investigated. A self-agglutination reaction was observed with two *S. uberis* strains (Table 5).

Table 5. Lectin agglutination reactions of *S. uberis* and *S. parauberis* with the lectins of *Helix pomatia* and *Dolichos biflorus*

Species	n	<i>Helix pomatia</i>	<i>Dolichos biflorus</i>	Self-agglutination
<i>S. uberis</i>	130	43* (32.5%)	0 (0%)	2 (1.5%)
<i>S. parauberis</i>	1	0 (0%)	0 (0%)	0 (0%)
<i>S. uberis</i> NCDO 2038, NCDO 2086 <i>S. parauberis</i> NCDO 2020, 94/16	4	0 (0%)	0 (0%)	0 (0%)

n = Number of strains

* = Number of positive strains (%)

Hemagglutination reactions were examined on glass slides using the group B streptococcal strains 395/2 and G28 as positive and negative controls. Compared to the positive reaction of the group B streptococcal strain 395/2 all *S. uberis* and *S. parauberis* investigated, also including the reference strains of both species, were negative.

A synergistic hemolytic reaction within the zone of staphylococcal β -toxin could be observed for five (3.8%) *S. uberis* strains (Fig. 4). The remaining *S. uberis* and *S. parauberis* strains were negative.

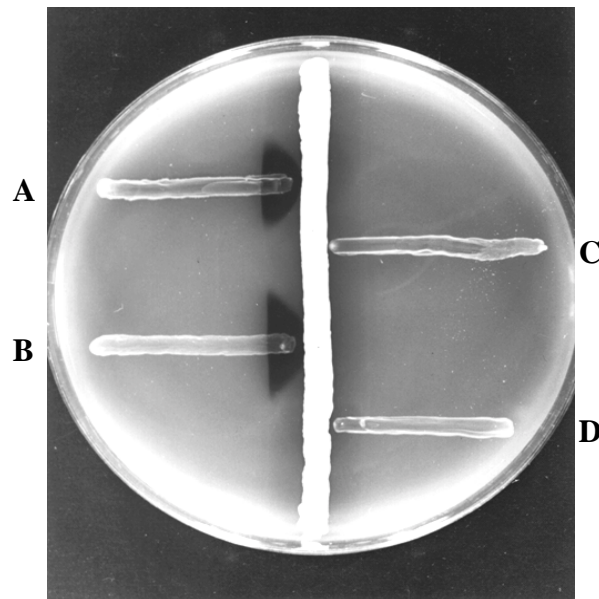


Figure 4. CAMP reaction of *S. uberis*. Vertical streak: β -hemolytic *S. aureus*. Horizontal streak: Phenotypically positive *S. uberis* (A) and *S. agalactiae* (B), and phenotypically negative *S. uberis* (C) and *S. parauberis* (D).

The different growth patterns of the 130 *S. uberis* strains, the one *S. parauberis* and the four reference strains were recorded after inoculation of the cultures in fluid media (3.3.7). Among the bacteria investigated, seven (5.4%) *S. uberis* strains and one *S. parauberis* reference strain grew as granular sediment with clear supernatant, while the remaining 123 (94.6%) *S. uberis* strains, the one (100%) *S. parauberis* strain and two *S. uberis* and one *S. parauberis* reference strains grew with a uniform turbidity of the growth media (Table 6).

The bacteria were additionally cultivated in soft agar (3.5.3). In soft agar six (86%) *S. uberis* strains as well as one *S. uberis* and one *S. parauberis* reference strain showed compact colonies. In contrast, 100 (80%) of the *S. uberis* strains, the one (100%) *S. parauberis* strain as well as one *S. uberis* and one *S. parauberis* reference strain developed a diffuse colony morphology. Among the *S. uberis* 11 strains (10%) developed a compact as well as a diffuse colony morphology. All three growth patterns are shown in fig. 5.

The relationship between growth patterns in fluid media and soft agar of both streptococcal species is summarized in table 6. Among the seven *S. uberis* strains with sediment appearance in fluid media, six strains (86%) showed compact colony morphology in soft agar, while one strain (14%) showed a diffuse colony morphology. The 123 *S. uberis* strains showing a uniform turbidity of the growth media appeared with compact (10%), diffuse (80%) as well as with compact and diffuse (10%) colonies in soft agar. The turbid-growing *S. parauberis* strain and one *S. uberis* reference strain showed a diffuse colony morphology. One turbid-growing *S. uberis* and one *S. parauberis* strain with growth as sediment had a compact colony morphology in soft agar (Table 6).

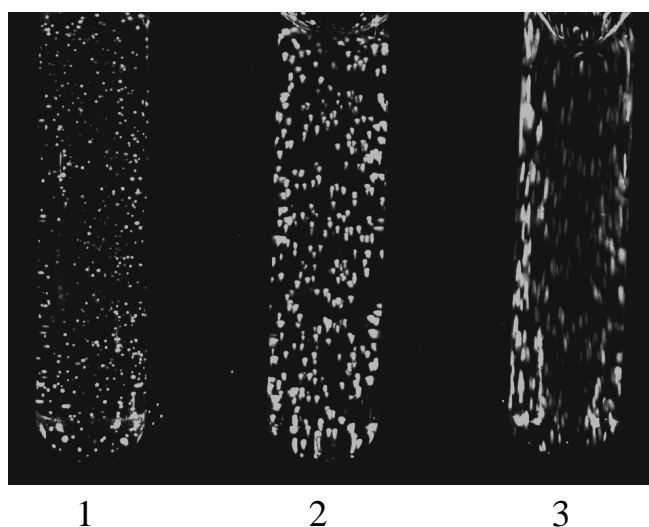


Figure 5. Characteristic growth patterns of *S. uberis* and *S. parauberis* in soft agar after 18-24 h incubation at 37°C. Typical compact (1), diffuse and compact (2) and diffuse (3) colony morphology.

Table 6. Growth pattern of 130 *S. uberis*, one *S. parauberis* and four reference strains of *S. uberis* and *S. parauberis* in fluid medium and soft agar

Growth pattern in fluid medium <i>S. uberis</i>	Colony morphology in soft agar		
	Compact	Diffuse	Compact/Diffuse
Sediment (n = 7)	6* (86%)	1 (14%)	0 (0%)
Turbid (n = 123)	12 (10%)	100 (80%)	11 (10%)
Growth pattern in fluid medium <i>S. parauberis</i>	Colony morphology in soft agar		
	Compact	Diffuse	Compact/Diffuse
Turbid (n = 1)	0 (0%)	1 (100%)	0 (0%)
Growth pattern in fluid medium <i>S. uberis</i> NCDO 2038, NCDO 2086	Colony morphology in soft agar		
	Compact	Diffuse	Compact/Diffuse
Sediment (n = 0)	(0%)	0 (0%)	0 (0%)
Turbid (n = 2)	1 (50%)	1 (50%)	0 (0%)
Growth pattern in fluid medium <i>S. parauberis</i> NCDO 2020, 94/16	Colony morphology in soft agar		
	Compact	Diffuse	Compact/Diffuse
Sediment (n = 1)	1 (50%)	0 (0%)	0 (0%)
Turbid (n = 1)	0 (0%)	1 (50%)	0 (0%)

n = Number of strains investigated

* = Number of strains with the respective growth pattern (%)

The 130 *S. uberis* strains, the one *S. parauberis* and the four reference strains of both streptococcal species were also investigated for salt aggregation by using different concentrations of ammonium sulfate. Among the 130 strains of *S. uberis* 25 strains (19%) and one reference strain of *S. uberis* exhibited positive aggregation reactions with ammonium sulfate concentrations ranging from 3.4 to 2.4 mol/l, 11 strains (8%) with ammonium sulfate concentrations ranging from ≤ 2.0 to 1.8 mol/l and 15 strains (12%) with ammonium sulfate concentrations ranging from ≤ 1.6 to 0.2 mol/l, respectively. The remaining 70 *S. uberis* strains (54%) and the *S. parauberis* strain, including one *S. uberis* and two *S. parauberis* reference strains were negative for all the used ammonium sulfate concentrations. A self-aggregation reaction could be observed for nine *S. uberis* strains (7%). The data are shown in table 7.

Table 7. Salt aggregation test of *S. uberis* and *S. parauberis* strains

Species	n	Salt aggregation				Self-aggregation
		SAT +			SAT -	
		≤ 1.6	≤ 2.0	≥ 2.4		
<i>S. uberis</i>	130	15* (12%)	11 (8%)	25 (19%)	70 (54%)	9 (7%)
<i>S. parauberis</i>	1	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)
<i>S. uberis</i> NCDO 2038, NCDO 2086	2	0 (0%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)
<i>S. parauberis</i> NCDO 2020, 94/16	2	0 (0%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)

n = Number of strains investigated

* = Number of positive strains (%)

SAT + = Aggregation in different concentrations of ammonium sulfate (mol/l)

SAT - = No aggregation in all concentrations of ammonium sulfate (mol/l)

The antibiotic sensitivity of the 130 *S. uberis* strains, the one *S. parauberis* strain and the four reference strains of *S. uberis* and *S. parauberis* are shown in table 8 and 9, respectively. All 130 *S. uberis* and two *S. uberis* reference strains were sensitive to penicillin-G, while 127 (96%), 107 (81%), 105 (80%) and 100 (76%) strains were sensitive to cefotaxim, erythromycin, cefacetil and clindamycin, respectively. Among the 132 strains 120 (91%) were resistant to colistin. With gentamicin, minocyclin, sulphamethoxazole/trimethoprim (SXT) and tetracycline, the *S. uberis* strains were sensitive (49%, 73%, 50%, 60%), intermediate (34%, 16%, 11%, 11%), and resistant (17%, 11%, 39%, 29%), respectively.

Table 8. Antibiotic sensitivity of 130 *S. uberis* strains and two *S. uberis* reference strains

Antibiotics	Sensitive	Intermediate	Resistant
Cefacetil	105* (80%)	20 (15%)	7 (5%)
Cefotaxim	127 (96%)	5 (4%)	0 (0%)
Clindamycin	100 (76%)	3 (2%)	29 (22%)
Colistin	7 (5%)	5 (4%)	120 (91%)
Erythromycin	107 (81%)	0 (0%)	25 (19%)
Gentamicin	64 (49%)	45 (34%)	23 (17%)
Minocycline	95 (73%)	22 (16%)	15 (11%)
Penicillin-G	132 (100%)	0 (0%)	0 (0%)
Sulphamethoxazole and Trimethoprim	66 (50%)	15 (11%)	51 (39%)
Tetracycline	79 (60%)	15 (11%)	38 (29%)

* = Number of strains (%)

One *S. parauberis* strain and two *S. parauberis* reference strains were sensitive to cefotaxim, colistin, minocyclin, penicillin-G and tetracycline, two strains sensitive to cefacetil, clindamycin, erythromycin and gentamicin, respectively. With sulphamethoxazole/trimethoprim (SXT), one *S. parauberis* was sensitive, one intermediate and one resistant.

Table 9. Antibiotic sensitivity of one *S. parauberis* strain and two *S. parauberis* reference strains

Antibiotics	Sensitive	Intermediate	Resistant
Cefacetil	2* (67%)	0 (0%)	1 (33%)
Cefotaxim	3 (100%)	0 (0%)	0 (0%)
Clindamycin	2 (67%)	0 (0%)	1 (33%)
Colistin	3 (100%)	0 (0%)	0 (0%)
Erythromycin	2 (67%)	1 (33%)	0 (0%)
Gentamicin	2 (67%)	1 (33%)	0 (0%)
Minocyclin	3 (100%)	0 (0%)	0 (0%)
Penicillin-G	3 (100%)	0 (0%)	0 (0%)
Sulphamethoxazole and Trimethoprim	1 (33%)	1 (33%)	1 (33%)
Tetracycline	3 (100%)	0 (0%)	0 (0%)

* = Number of strains (%)

4.2 Molecular Characterization

The 16S rRNA gene of the 130 *S. uberis* strains, the one *S. parauberis* strain and the four reference strains of both species was successfully amplified by PCR. The size of the amplicon of both streptococcal species was 1430 bp relative to DNA size marker (Fig. 6).

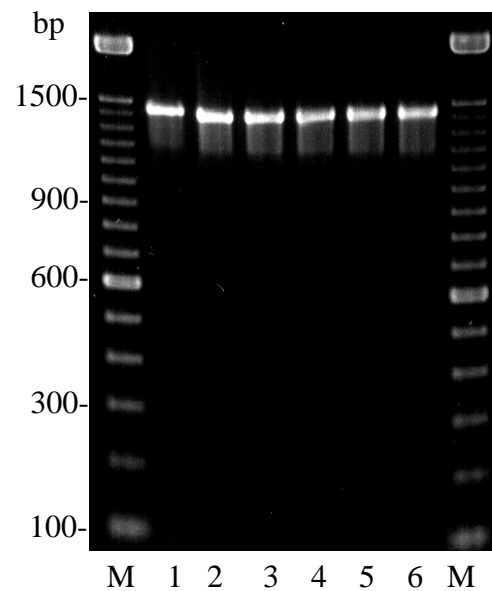


Figure 6. Amplicons of the 16S rRNA gene of *S. uberis* (1, 2, 3) and *S. parauberis* (4, 5, 6) with a size of 1430 bp using the oligonucleotide primers ARI and AMII. M = a 100 bp ladder served as size marker.

The amplified 16S rRNA fragment was digested with the restriction endonucleases *RsaI*, *AvaII* and *MspI*, respectively. In all *S. uberis* strains (n = 132) the restriction fragment length polymorphism (RFLP) after *RsaI* restriction revealed the same four fragments with a size of approx. 140, 190, 220 and 700 bp, respectively. Predicted fragment sizes from sequence (accession number U41048) for *S. uberis* were 8, 40, 146, 143, 183, 212 and 700 bp. *RsaI* RFLP analysis of the *S. parauberis* strains yielded fragment sizes of approx. 140, 190, 380 and 700 bp, respectively (Fig. 7).

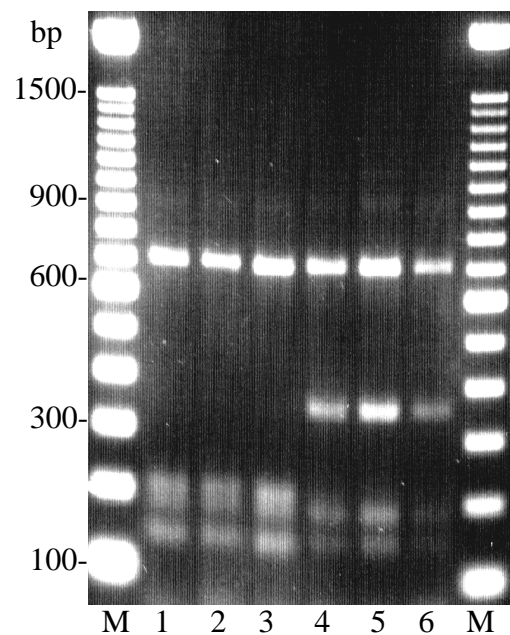


Figure 7. Typical fragments of the PCR amplified 16S rRNA gene of *S. uberis* (1, 2, 3) and *S. parauberis* (4, 5, 6) after digestion with the restriction enzyme *RsaI*. M = see fig. 6.

RFLP after *Ava*II restriction revealed three different fragments with a size of 230, 310 and 900 bp, respectively. *Ava*II RFLP analysis of the *S. parauberis* strains yielded identical fragment sizes of 230 and 1,200 bp, respectively (Fig. 8).

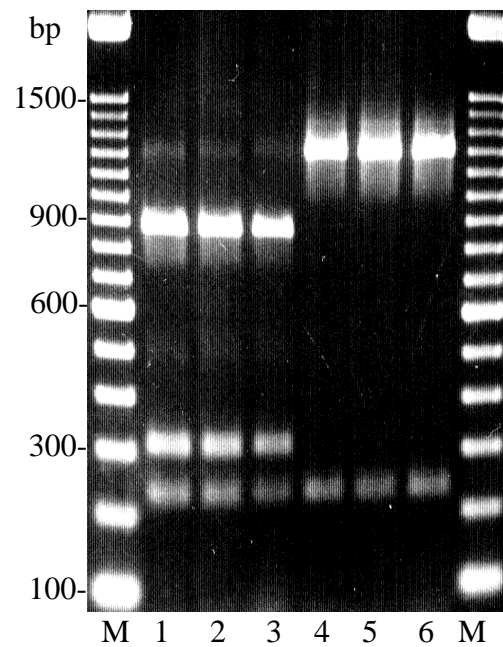


Figure 8. Typical fragments of the PCR amplified 16S rRNA gene of *S. uberis* (1, 2, 3) and *S. parauberis* (4, 5, 6) after digestion with the restriction enzyme *Ava*II. M = see fig. 6.

RFLP after *MspI* restriction revealed five fragments with a size of 100, 140, 170, 310 and 570 bp, respectively. *MspI* RFLP analysis of the *S. parauberis* strains also yielded fragment sizes of 100, 140, 170, 310 and 570 bp, respectively (Fig. 9).

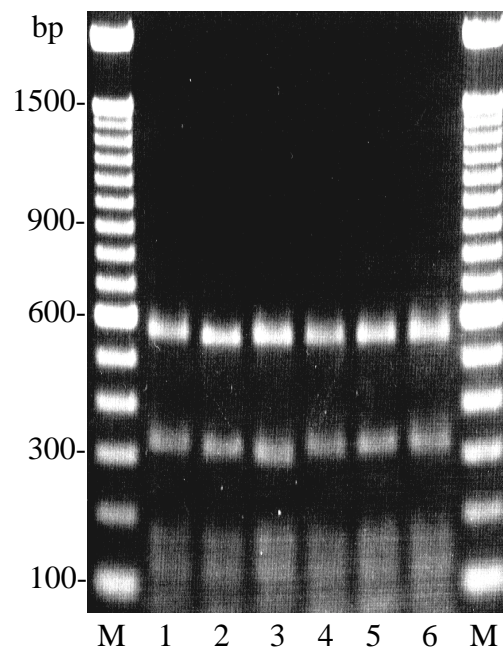


Figure 9. Typical fragments of the PCR amplified 16S rRNA gene of *S. uberis* (1, 2, 3) and *S. parauberis* (4, 5, 6) after digestion with the restriction enzyme *MspI*. M = see fig. 6.

Using the primer pair ub-I and ub-II (3.6.6) a species specific part of the 16S rRNA gene of 130 *S. uberis* and two *S. uberis* reference strains could be amplified. The specific amplicon had a size of 440 bp (Fig. 10). The *S. parauberis* strains investigated were negative.

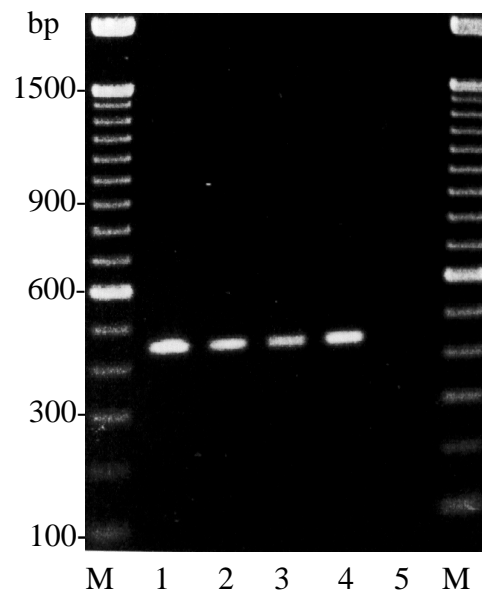


Figure 10. Amplicons of *S. uberis* (1, 2, 3, 4) with a size of 440 bp using the *S. uberis* 16S rRNA gene specific oligonucleotide primers ub-I and ub-II; *S. parauberis* (5) served as negative control. M = see fig. 6.

Using the primer pair paraub-I and paraub-II (3.6.6), a *S. parauberis* specific part of the 16S rRNA gene could be amplified for all three *S. parauberis* strains, also including the reference strains. The specific amplicon had a size of 880 bp (Fig. 11). All 130 *S. uberis* and the two *S. uberis* reference strains investigated were negative.

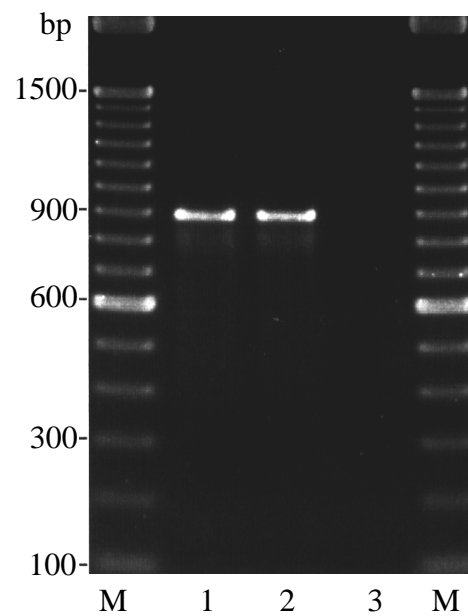


Figure 11. Amplicons of *S. parauberis* (1, 2) with a size of 880 bp using the *S. parauberis* 16S rRNA gene specific oligonucleotide primers paraub-I and paraub-II; *S. uberis* (3) served as negative control. M = see fig. 6.

A successful amplification of a *S. uberis* specific part of the 16S-23S rDNA intergenic spacer region could be performed for all 130 *S. uberis* and the two *S. uberis* reference strains by using the specific primer pair STRU-UbI and STRU-UbII (3.6.7). A specific amplicon with a size of 340 bp was obtained (Fig. 12). The three *S. parauberis* strains were negative.

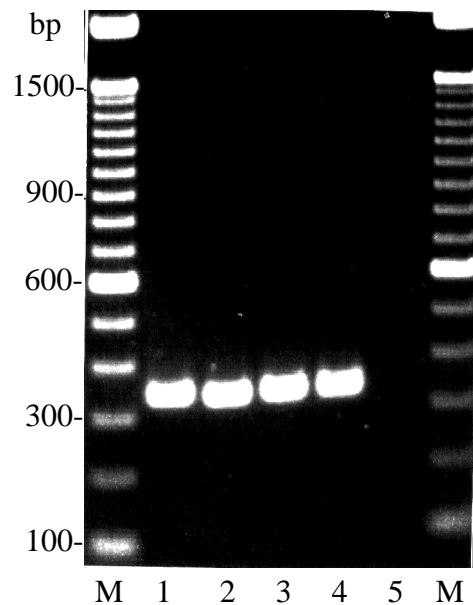


Figure 12. Amplicons of *S. uberis* (1, 2, 3, 4) with a size of 340 bp using the *S. uberis* 16S-23S rDNA intergenic spacer region specific oligonucleotide primers STRU-UbI and STRU-UbII; *S. parauberis* (5) served as negative control. M = see fig. 6.

Using the primer pair paraub-16S-23S-I and paraub-16S-23S-II (3.6.7), a *S. parauberis* specific part of the 16S-23S rDNA intergenic spacer region could be amplified for all three *S. parauberis* strains investigated. The amplicon had a size of 200 bp (Fig. 13). All 130 *S. uberis* and two reference strains of *S. uberis* examined were negative.

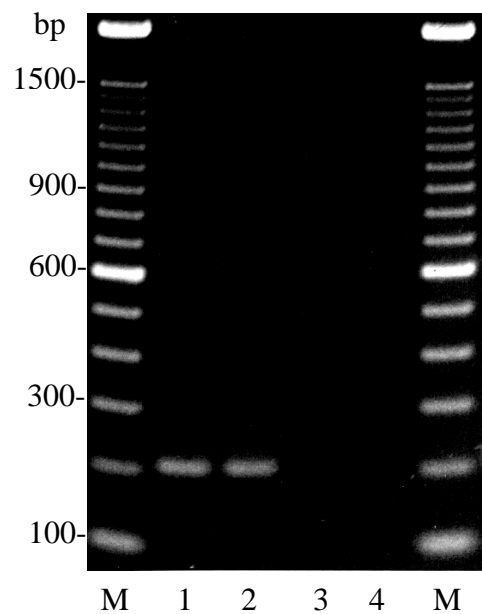


Figure 13. Amplicons of *S. parauberis* (1, 2) with a size of 200 bp using the *S. parauberis* 16S-23S rDNA intergenic spacer region specific oligonucleotide primers paraub-16S-23S-I and paraub-16S-23S-II; *S. uberis* (3, 4) served as negative control. M = see fig. 6.

A species-specific part of the 23S rRNA gene of *S. uberis* could successfully be amplified for all 130 *S. uberis* and the two *S. uberis* reference strains by using the primer pair ub-23S-I and ub-23S-II (3.6.8). The specific amplicon had a size of 450 bp (Fig. 14). The three *S. parauberis* strains investigated were negative.

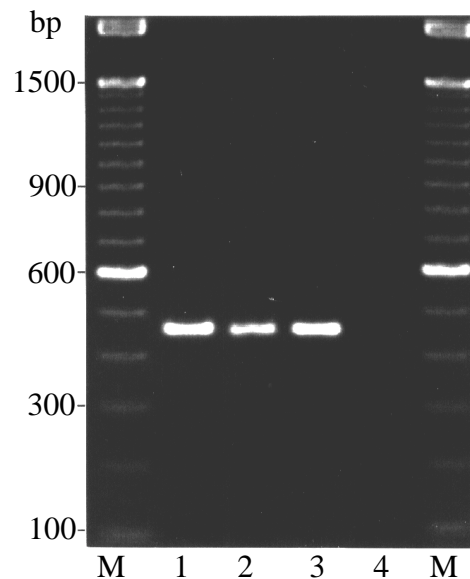


Figure 14. Amplicons of *S. uberis* (1, 2, 3) with a size of 450 bp using the *S. uberis* 23S rRNA gene specific primers ub-23S-I and ub-23S-II; *S. parauberis* (4) served as negative control. M = see fig. 6.

A species-specific part of the 23S rRNA gene of *S. parauberis* could effectively be amplified for all three investigated strains by using primer pair paraub-23S-I and paraub-23S-II (3.6.8). The specific amplicon had a size of 480 bp (Fig. 15). All 130 *S. uberis* and both *S. uberis* reference strains were negative.

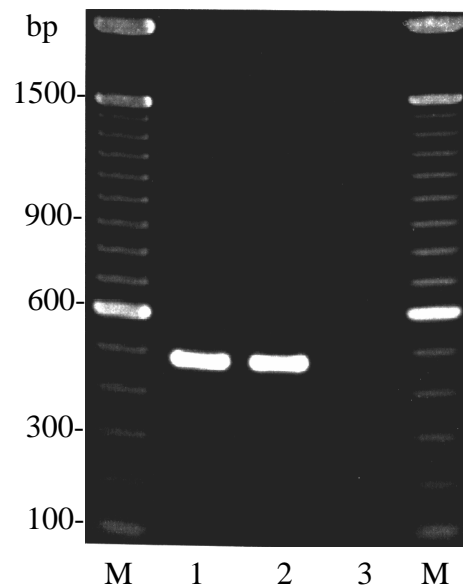


Figure 15. Amplicons of *S. parauberis* (1, 2) with a size of 480 bp using the *S. parauberis* 23S rRNA gene specific primers paraub-23S-I and paraub-23S-II; *S. uberis* (3) served as negative control. M = see fig. 6.

Using the primer pair CAMP ub-I and CAMP ub-II (3.6.9) the CAMP factor gene *cfu* could be amplified for 5 *S. uberis*. The amplified product had a size of 870 bp. All five genotypically CAMP positive *S. uberis* were also phenotypically CAMP positive. Selected phenotypically CAMP negative *S. uberis* (n = 31) and all three phenotypically CAMP negative *S. parauberis* strains were negative (Fig. 16).

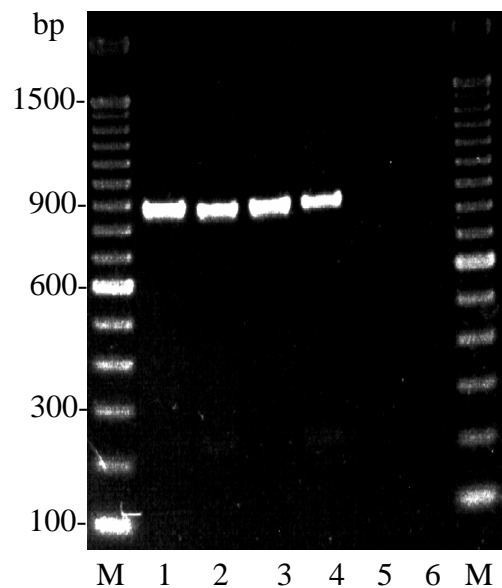


Figure 16. Amplicons of *S. uberis* (1, 2, 3, 4) with a size of 870 bp using the *S. uberis* CAMP factor gene *cfu* specific primers CAMP ub-I and CAMP ub-II. A phenotypically CAMP negative *S. uberis* (5) and a phenotypically CAMP negative *S. parauberis* (6) served as negative control. M = see fig. 6.

In parallel, the CAMP factor gene *cfu* was amplified using the primer pair *cfu*-I and *cfu*-II (3.6.9). With this primer pair the same five phenotypically and genotypically CAMP positive strains mentioned before were positive showing an amplicon with a size of 680 bp (Fig. 17). Selected phenotypically CAMP negative *S. uberis* (n = 31) and all three phenotypically CAMP negative *S. parauberis* strains were negative.

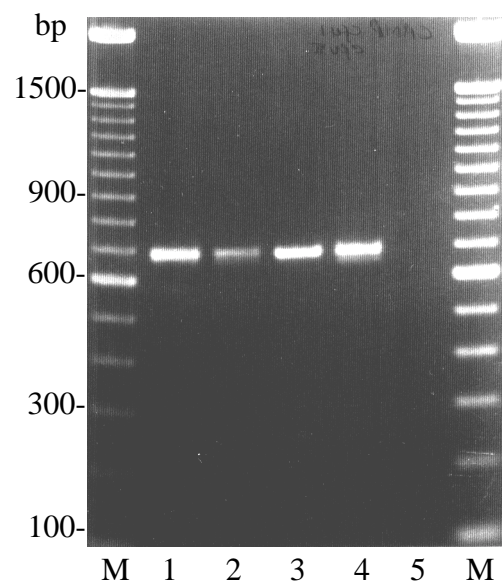


Figure 17. Amplicons of *S. uberis* (1, 2, 3, 4) with a size of 680 bp using the *S. uberis* CAMP factor gene *cfu* specific primers *cfu*-I and *cfu*-II. A phenotypically CAMP negative *S. uberis* (5) served as negative control. M = see fig. 6.

The nucleotide sequences of both the *skc* and *pauA* genes obtained from data bank (accession number AJ006413; Johnsen et al. 1999 and accession number AJ012548; Rosey et al. 1999) were aligned and compared with the help of computer program Meg Align 1993-1997 (DNASTAR Inc., Konstanz, Germany). Nucleotide sequence AJ012548 lacked 115 nucleotides upstream of *pauA*, while nucleotide sequence AJ006413 was 114 nucleotides shorter downstream of *skc*. This is shown in fig. 18. Both sequences showed a high degree of sequence homology.

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1          SKC-I                                     60
1  CTCCTCTCCA ACAAGAGGG CTTTTTAAAG ATAAAATCAA TAATTTTAC CATAAATGGT
2  -----
61
1  ATTTTTGTC TAGTTCITTT TTATCGGAAT CCGTTAAAAA ATGACATTAA TATCTACTTC
2  -----ATTAA TATCTACTTC
121
1  AATTTGTTCT AAAATGAAAT TATATTCAC TCTGTTACAT AACTTTGTGA TTATTTAGGA
2  AATTTGTTCT AAAATGAAAT TATATTCAC TCTGTTACAT AACTTTGTGA TTATTTAGGA
181
1  TAAAATAAGG GAGATTTTTA TGAAAAAATG GTTTTTAATA TTAATGCTTT TGGGAATATT
2  TAAAATAAGG GAGATTTTTA TGAAAAAATG GTTTTTAATA TTAATGCTTT TGGGAATATT
241
1  TGGTTGTGCT ACTCAACCAT CAAAGGTTGC AGCAATAACC P 38 GGTATGATT CCGACTACTA
2  TGGTTGTGCT ACTCAACCAT CAAAGGTTGC AGCAATAACC GGTATGATT CCGACTACTA
301
1  CGCTAGATAT ATTGATCCCG ATGAAAATAA AATAACATTT GCCATAAATG TTGATGGTTT
2  CGCTAGATAT ATTGATCCCG ATGAAAATAA AATAACATTT GCCATAAATG TTGATGGTTT
361
1  TGTCGAAGGT AGTAATCAAG AAATCCTTAT TAGAGGAATT CATCATGTTT TAACAGATCA
2  TGTCGAAGGT AGTAATCAAG AAATCCTTAT TAGAGGAATT CATCATGTTT TAACAATCA
421
1  AAACCAAAAG ATTGCTACAA AGGCCGAGTT GTTAGACGCT ATTAGACATC AAATGGTTCT
2  AAACCAAAAG ATTGCTACAA AGGCCGAGTT GTTAGACGCT ATTAGACATC AAATGGTTCT
481
1  TCTACAATTG GATTATTCCT ATGAACTAGT CGACTTTGCG CCTGATGCAC AATTATTAAC
2  TCTACAATTG GATTATTCCT ATGAACTAGT CGACTTTGCG CCTGATGCAC AATTATTAAC
541
1  ACGAGATCGA CGGCTTTTAT TTGCCAATCG AAATTTTGAG GAATCCGTAT CACTTGAAGA
2  ACGAGATCGA CGGCTTTTAT TTGCCAATCG AAATTTTGAG GAATCCGTAT CACTTGAAGA
601
1  TACTATTCAA GAATACCTTT TAAAAGGGCA TGTATTCTC AGAAAACGGG TTGAAGAACC
2  TACTATTCAA GAATACCTTT TAAAAGGGCA TGTATTCTC AGAAAACGGG TTGAAGAACC
661
1  TATCACTCAT CCTACTGAGA CTGCTAATAT TGAGTATAAA GTTCAATTCG CACTAAAGA
2  TATCACTCAT CCTACTGAGA CTGCTAATAT TGAGTATAAA GTTCAATTCG CACTAAAGA
721
1  TGGGGAATTC CACCCACTAC CTATTTTTGT AGACTACGGA GAAAAACATA TTGGAGAAAA
2  TGGGGAATTC CACCCACTAC CTATTTTTGT AGACTACGGA GAAAAACATA TTGGAGAAAA

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781
1 ATTAACCTCT GACGAGTTTC GAAAAATTGC AGAAGAAAAG CTTTGTCAAC GCTACCCTGA
2 ATTAACCTCT GACGAGTTTC GAAAAATTGC AGAAGAAAAG CTTTGTCAAC GCTACCCTGA
841
1 CTATATGATT GATCAAAAAG AATATACTAT CATTAACAC AATTCTCTTG GTCAACTTCC
2 CTATATGATT GATCAAAAAG AATATACTAT CATTAACAC AATTCTCTTG GTCAACTTCC
901
1 AAGATATTAT TCTTATCCAG ATGATTTTCAG CTACGAAATT CAAGATAGGC AACGTATCAT
2 AAGATATTAT TCTTATCCAG ATGATTTTCAG CTACGAAATT CAAGATAGGC AACGTATCAT
961
1 GGCTAAGGAC CCAAAGTCCG GAAAAGAACT CGGTGAAACT CAAAGTATTG ATAATGTTTT
2 GGCTAAGGAC CCAAAGTCCG GAAAAGAACT CGGTGAAACT CAAAGTATTG ATAATGTTTT
1021
1 TGAGAAATAC CTTATTACCA AAAAAAGTTA TAAACCTTAA ATGAAGTATC CATTAATTTT
2 TGAGAAATAC CTTATTACCA AAAAAAGTTA TAAACCTTAA ATGAAGTATC CATTAATTTT
1081
1 TTCTGCATCA CTAAAAAAC ACGAGCATCA CTTTCAAAGG GGAAGGCCTT C-----
2 TTCTGCATCA CTAAAAAAC ACGAGCATCA CTTTCAAAGG G-AAGGCCTT CGTTAGTAAC
1141
1 -----
2 GCTAGTGTTC TTATTTTATT TTTTCTTTA AATCAAACAA AACAGTCATA GCTTCCATTG
1201
1 -----
2 GAGTCATATT CATGACATCT ACTTCTTTAA GTCTATTAAT GATATC
1246
1 ----- S. uberis skc
2 ----- S. uberis pauA

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Figure 18. Sequence alignment of *skc* and *pauA* genes and flanking regions of *S. uberis* (accession number AJ006413; Johnsen et al. 1999 and accession number AJ012548; Rosey et al. 1999). The oligonucleotide primers of both gene sequences used are underlined; the marked areas indicate the differences in nucleotide sequences.

Investigating the 130 *S. uberis*, the one *S. parauberis* and the four reference strains of both species for the *skc* gene a specific amplicon with a size of 1130 bp could be observed for 128 *S. uberis* strains. The remaining *S. uberis* and the three *S. parauberis* strains were negative (Fig. 19).

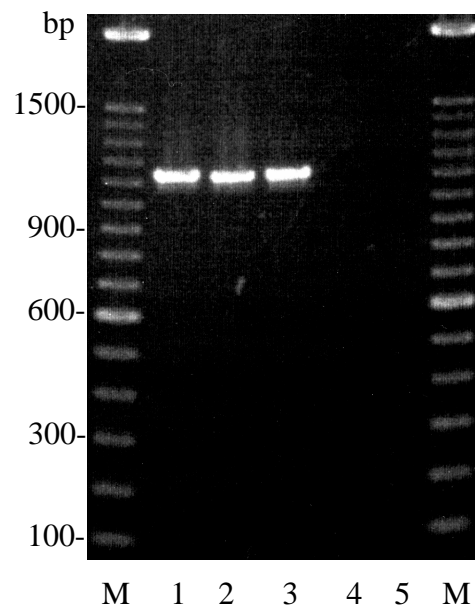


Figure 19. Amplicons of *S. uberis* (1, 2, 3) with a size of 1130 bp using the *S. uberis skc* gene specific primers SKC-I and SKC-II; *skc* gene negative *S. uberis* and *S. parauberis* are shown in lane 4 and 5. M = see fig. 6.

Investigating the strains for *pauA* gene a specific amplicon with a size of 800 bp could be observed for all 128 *skc* gene positive *S. uberis* strains. No amplicon could be observed for the remaining four *skc* gene negative *S. uberis* strains and the three *skc* gene negative *S. parauberis* strains (Fig. 20).

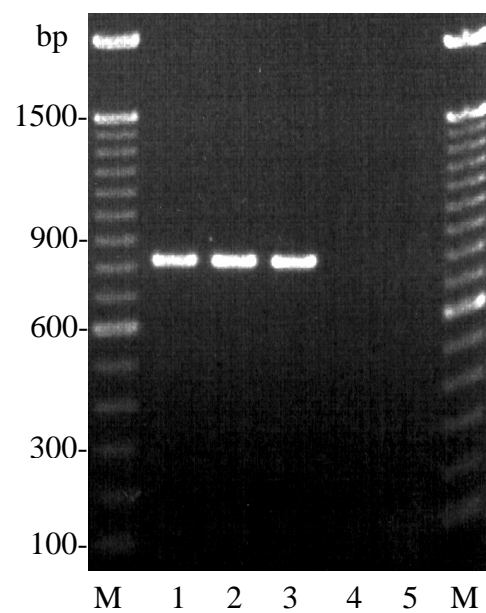


Figure 20. Amplicons of *S. uberis* (1, 2, 3) with a size of 800 bp using the *S. uberis* *pauA* gene specific primers P38 and P39; *pauA* gene negative *S. uberis* and *S. parauberis* are shown in lane 4 and 5. M = see fig. 6.

For DNA fingerprinting the *S. uberis* a macrorestriction analysis of their chromosomal DNA was determined by pulsed-field gel electrophoresis (PFGE). This was performed with 69 arbitrarily selected strains from 57 cows of 26 different farms (Fig. 21). Digestion of the chromosomal DNA of the isolates was performed with the endonuclease *SmaI*, while 22 strains were additionally digested with the endonuclease *ApaI*.

The PFGE patterns obtained after digestion with the endonuclease *SmaI* produced 8-13 distinctive fragments using the selected pulsed-field program, compared to the endonuclease *ApaI* producing 7-11 distinctive fragments.

According to the DNA fingerprinting results, nonidentical as well as identical PFGE patterns could be observed for some of the isolates within different quarters of an individual cow and different cows within the same farm. Among the 69 strains from 57 cows from 26 different farms investigated, 55 different DNA patterns were observed.

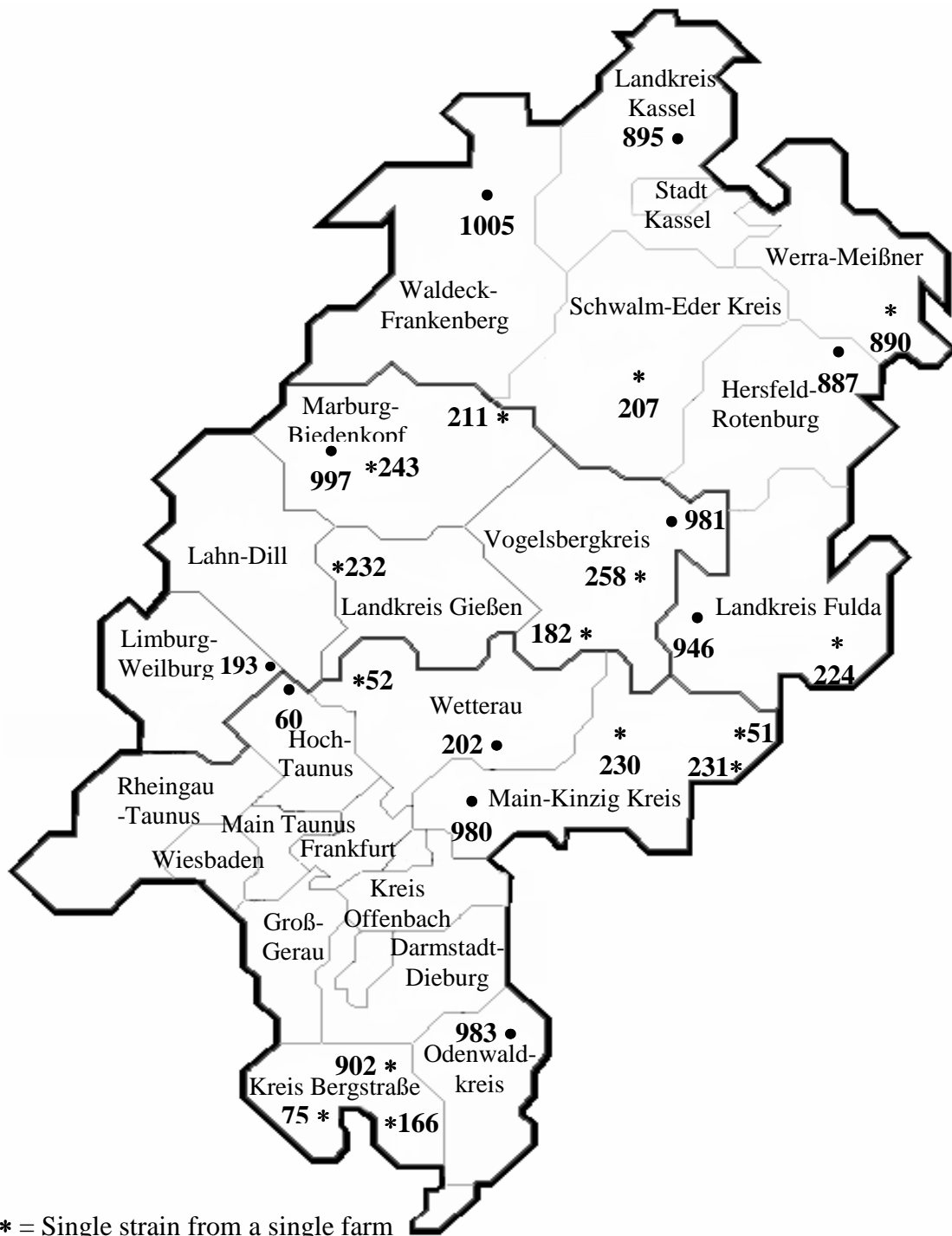


Figure 21. The location and code of the 26 farms from which the 69 *S. uberis* strains were selected for macrorestriction analysis of chromosomal DNA by pulsed-field gel electrophoresis.

As shown in fig. 22 and 23, the chromosomal DNA of nine *S. uberis* isolates from nine cows of farm 1 (887) were digested with the restriction endonucleases *SmaI* and *ApaI*, respectively. An identical *SmaI* PFGE pattern was noted in lane 1 and 5, lane 2 and 3 as well as in lane 6 and 8, respectively, the isolates of lane 4, 7 and 9 showed different PFGE patterns (Fig. 22).

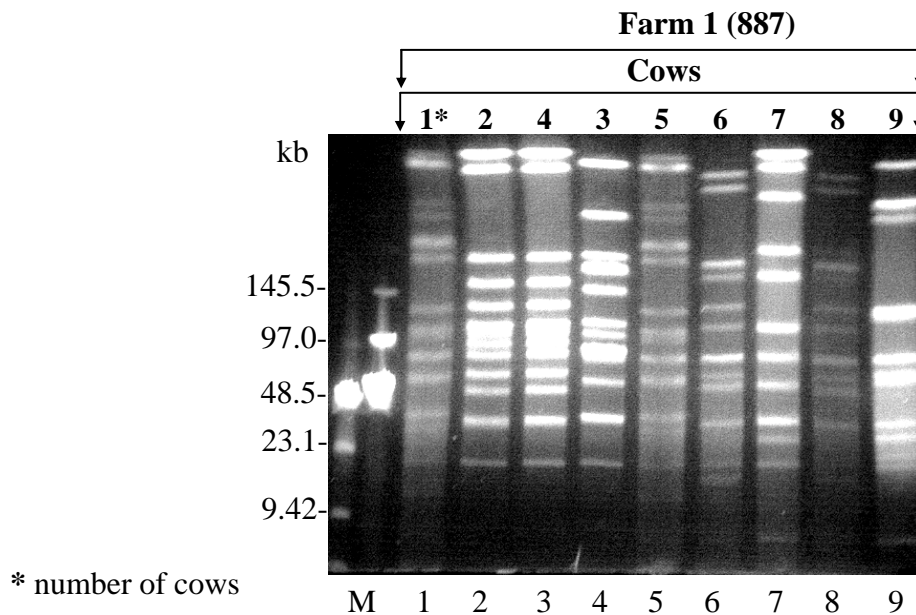


Figure 22. Pulsed-field gel electrophoretic restriction patterns of chromosomal DNA of nine *S. uberis* isolated from nine different cows of a single farm using the restriction enzyme *SmaI* with DNA restriction pattern I (lane 1 and 5), pattern II (lane 2 and 3), pattern III (lane 4), pattern IV (lane 6 and 8), pattern V (lane 7), pattern VI (lane 9). M = low range PFG (0.1-200 kb) and lambda ladder PFG (50-1000kb) were used as marker.

Using the restriction endonuclease *ApaI*, the chromosomal DNA of the nine isolates also displayed an identical PFGE pattern for cow 1 and 5, cow 2 and 4 as well as cow 6 and 8, respectively, while isolates of cow 3, 7 and 8 exhibited nonidentical PFGE patterns (Fig. 23).

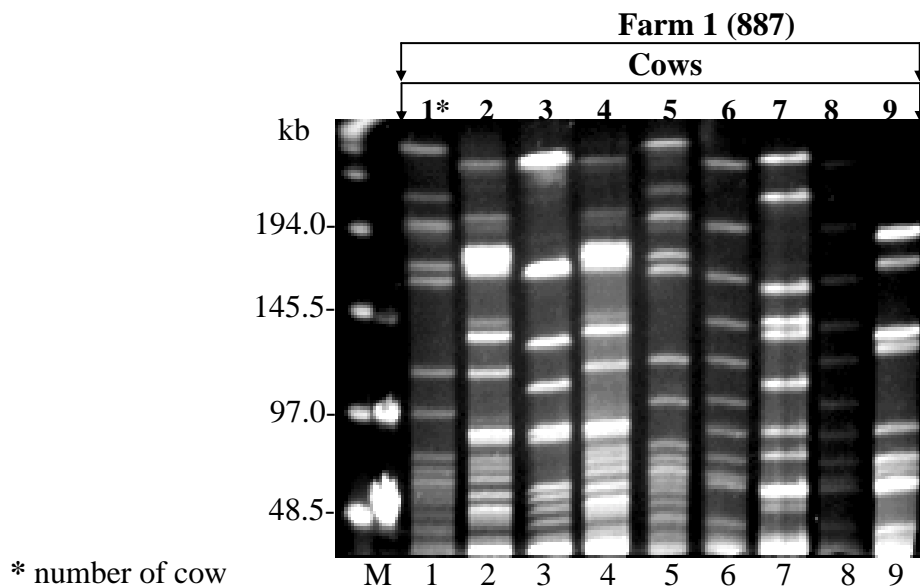
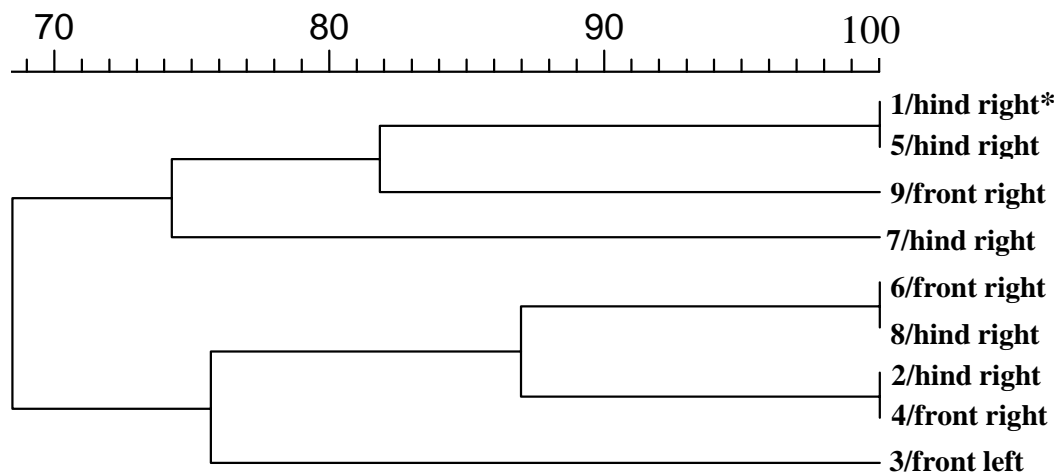


Figure 23. Pulsed-field gel electrophoretic restriction patterns of chromosomal DNA of nine *S. uberis* isolated from nine different cows of a single farm (see fig. 22) using the restriction enzyme *ApaI* with DNA restriction pattern I (lane 1 and 5), pattern II (lane 2 and 4), pattern III (lane 3), pattern IV (lane 6 and 8), pattern V (lane 7), pattern VI (lane 9). M = mentioned in fig. 22.

To determine the degree of similarity of the six different *SmaI* PFGE patterns, the nine tested strains were examined by dendrogram analysis (Fig. 24). The dendrogram results confirmed the identical as well as the nonidentical status of the different isolates of a single farm.



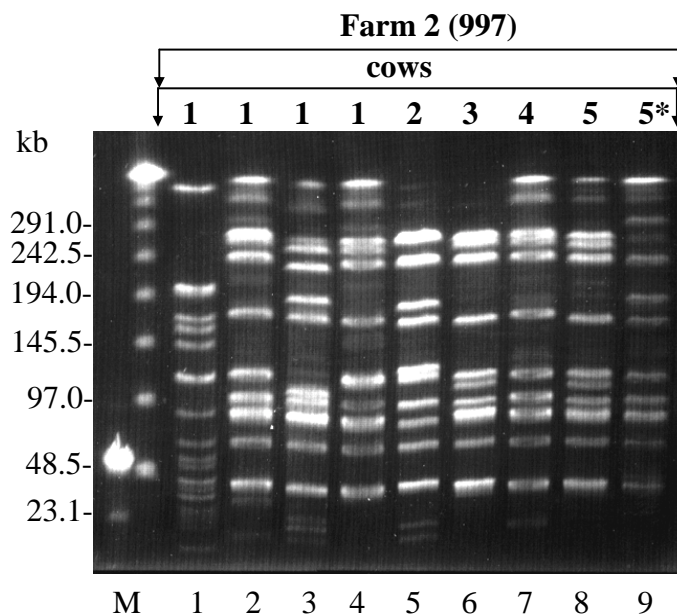
* cow number/ quarter

Figure 24. Dendrogram analysis of PFGE patterns (Fig. 22) of chromosomal DNA of nine *S. uberis* isolated from a single farm 1 (887) using the restriction enzyme *SmaI*.

The PFGE patterns of 12 isolates from six different cows of farm 2 (1997) are shown in fig. 25, 26. The results revealed an identity between isolates of different quarters of an individual cow and different cows within the same herd. A single PFGE pattern could be observed for five isolates of three cows and two isolates of two cows, respectively. The identical PFGE patterns are shown in lanes 2, 4, 7, 1 and 3 in fig. 25, 26 as well as in lanes 6 and 8 (Fig. 25), respectively. The five isolates from four cows in lanes 1, 3, 5, 9 and 2 (Fig. 25, 26) had diverse PFGE patterns and appeared to be nonidentical.

A dendrogram analysis of the seven PFGE pattern is shown in fig. 27.

Comparable results were obtained on farm 3 (1983) with five isolates from four different cows (Fig. 26). An identical PFGE pattern could be observed in two isolates of an individual cow, whereas different PFGE patterns were obtained from the other three isolates of three cows (Fig. 27).



* number of cows

Figure 25. Pulsed-field gel electrophoretic restriction patterns of chromosomal DNA of 9 *S. uberis* isolated from 5 cows of a single farm using the restriction enzyme *Sma*I with 5 different DNA restriction patterns; pattern I (lane 1), pattern II (lane 2, 4, 7), pattern III (lane 3), pattern IV (lane 5), pattern V (lane 6, 8) and pattern VI (lane 9). M = see fig. 22.

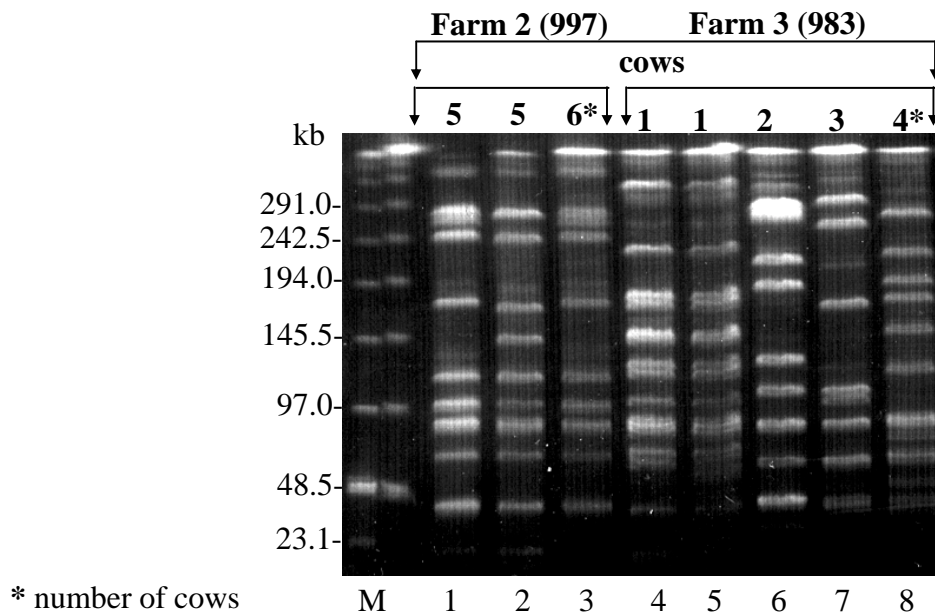
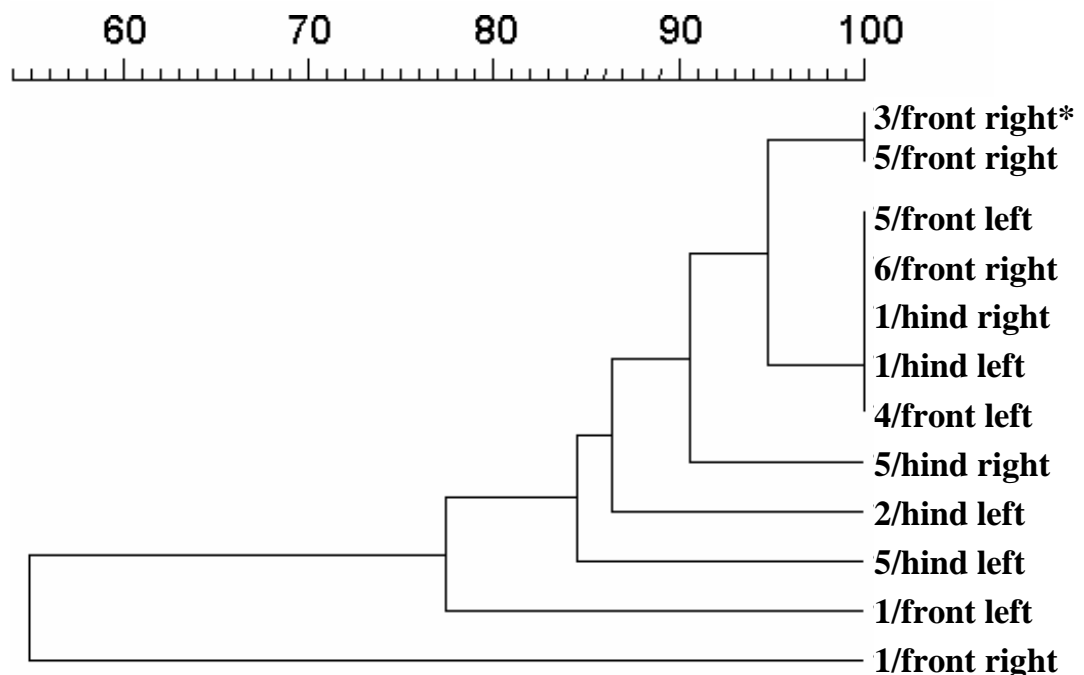


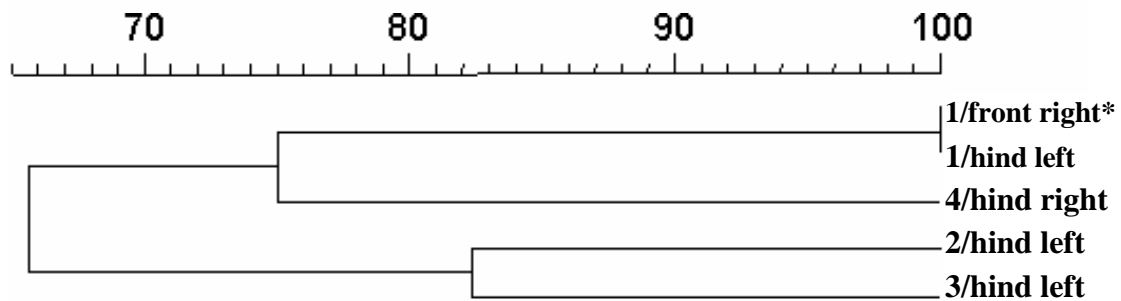
Figure 26. Pulsed-field gel electrophoretic restriction patterns of chromosomal DNA of 8 *S. uberis* isolated from 6 different cows of two different farms (farm 2 (Fig. 26), lane 1-3; farm 3, lane 4-8) using the restriction enzyme *Sma*I with different DNA restriction patterns; pattern II (lane 1, 3), pattern VII (lane 2); pattern I (lane 4, 5), pattern II (lane 6), pattern III (lane 7) and pattern IV (lane 8) fig. 26. M = see fig. 22.



* cow number/ quarter

Figure 27. Dendrogram analysis of *Sma*I PFGE patterns of chromosomal DNA of 12 *S. uberis* isolated from 6 different cows of farm 2 (1997) (Fig. 25, lane 1-9; Fig. 26, lane 1-3).

An additional dendrogram was developed for analysis of the four PFGE patterns of the four isolates of farm 3 (983) shown in fig. 28.



* cow number/ quarter

Figure 28. Dendrogram analysis of *SmaI* PFGE patterns of chromosomal DNA of five *S. uberis* isolated from four different cows of farm 3 (983) (Fig. 26, lane 4-8).

A summary of the *SmaI* and *ApaI* PFGE patterns of all 69 as well as 22 *S. uberis* isolates and a dendrogram of PFGE patterns are shown in table 11 and 12 and in fig. 29.

Table 11. Macrorestriction analysis of chromosomal DNA of arbitrarily selected 69 *S. uberis* strains digested with *Sma*I restriction endonuclease

Number and code of farm	Number of cows (n = 57)	Number of isolated strains (n = 69)	Number of restriction patterns (n = 55)
1 (887)	9	9	6
2 (997)	6	12	7
3 (983)	4	5	4
4 (895)	4	5	4
5 (980)	3	3	3
6 (193)	3	4	3
7 (202)	1	3	1
8 (258)	2	2	2
9 (1005)	3	3	3
10 (981)	2	2	2
11 (946)	1	2	1
12 (60)	5	5	5
14* farms	14	14	14
n = 26			

n = Total number

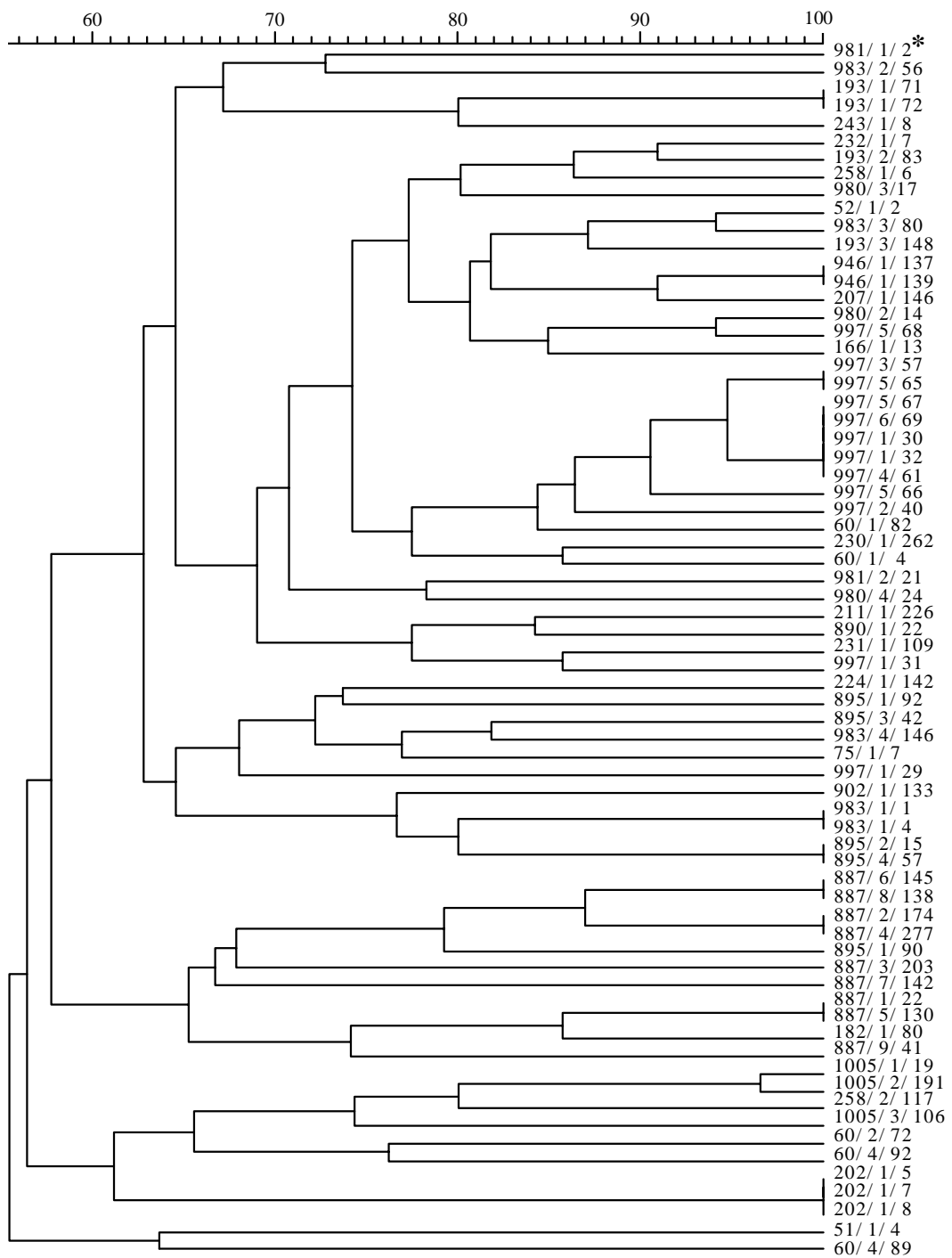
* = 14 single strains of 14 single farms with single PFGE patterns, respectively

Table 12. Macrorestriction analysis of chromosomal DNA of *S. uberis* strains digested with *Apa*I restriction endonuclease

Number and code of farm	Number of cows (n = 21)	Number of strains (n = 22)	Number of restriction patterns (n = 16)
1 (887)	9	9	6
2 (174)	8	9	6
4* farms	4	4	4
n = 7			

n = Total number

* = Four single strains of four single farms with single PFGE patterns, respectively



* Farm number/ cow number/ quarter number recorded by Veterinäruntersuchungsamt

Figure 29. Degree of similarity of 55 PFGE patterns of 69 *S. uberis* strains isolated from 57 different cows of 26 farms.

5. Discussion

S. uberis is important to the veterinary domain because of its increasing association with bovine mastitis. The *S. uberis* mastitis causes a tremendous economic loss in milk production and has become the major environmental mastitis agent of present time (Todhunter et al. 1994). On the basis of chromosomal DNA hybridizations Garvie and Bramley (1979) and Collins et al. (1984) suggested the existence of two distinct *S. uberis* genotypes. William and Collins (1990) designated the type II *S. uberis* as *S. parauberis*.

In the present investigation 130 *S. uberis* cultures were isolated, together with one *S. parauberis* culture, from bovine milk samples during three months of the winter season from a defined area. The isolates were studied together with two *S. uberis* and two *S. parauberis* reference strains. Almost all investigated cultures were isolated from subclinical mastitis, five strains were isolated from clinical mastitis. The present results strongly support the findings described by Lerondelle (1985) that a *S. uberis* infection rarely gives rise to clinical mastitis. The infection remains subclinical during long periods of time. In the absence of treatment this causes serious losses in milk production. Also corresponding to the present work, Bramley (1980) and Jayarao et al. (1999) described a high prevalence of subclinical forms of *S. uberis* intramammary infections in dairy cows. According to these authors, a *S. uberis* subclinical mastitis frequently occurs before parturition and near drying-off period, whereas a clinical mastitis with *S. uberis* could be observed more frequently in the first five weeks of lactation. In contrast a low number of subclinical mastitis could be recorded in that particular stage of lactation. However, in contrary to these results Wilson and Richards (1980) reported about a low prevalence of *S. uberis* subclinical mastitis. These authors examined British dairy herds and recorded subclinical mastitis caused by *S. uberis* in 1.5% of the quarters.

Among the 131 isolates investigated in the present study only one strain was identified as *S. parauberis*. Garvie and Bramley (1979) isolated *S. parauberis* strains

from milk samples of cows from British dairy herds. According to the present results the occurrence of *S. parauberis* as mastitis causing agent seems to be rare.

After cultivation of the isolates of the present study on sheep blood agar, the majority of the *S. uberis* strains produced an α -hemolysis, while few *S. uberis* strains exhibited a γ -hemolysis. This corresponded to the findings reported by Seeley 1951; Cullen 1967; 1968 and 1969, respectively. King (1981a) and later Hardie (1986) also described α -hemolytic as well as γ -hemolytic patterns for this species. Similarly, Obiger and Seeleman (1961) mentioned α -hemolytic patterns of *S. uberis* isolated from cases of mastitis and cattle tonsils. However, samples collected from swine tonsils exhibited β -hemolysis. Sharma (1969) reported about all three hemolytic patterns for *S. uberis*. However, the majority exhibited α -hemolysis, less than 10% γ -hemolysis and 2.5% β -hemolysis. In 1954, Seeleman reported that *S. uberis* could also appear β -hemolytic. In the studies of Hahn (1981) *S. uberis* failed to produce β -hemolysis, whereas the majority of the isolates exhibited a γ -hemolytic pattern, while few cultures were α -hemolytic. According to Bridge and Sneath (1983), *S. uberis* was α -hemolytic.

As far as *S. parauberis* is concerned, Garvie and Bramley (1979), Williams and Collins (1979) and later Collins et al. (1984) described that, after cultivation on sheep blood agar, *S. parauberis* appeared to be α -hemolytic or non-hemolytic. Doménech et al. (1996) cultured α -hemolytic *S. parauberis* strains isolated from diseased turbot. Corresponding to these results the three *S. parauberis* strains of the present study also exhibited an α -hemolysis.

Esculin degrading is a common property of some gram-positive cocci belonging to genus *Streptococcus* and of bacteria of genus *Enterococcus*. For this purpose an initial collection and cultivation of the isolates of the present investigation was performed on columbia esculin agar. On this media all 130 *S. uberis*, the one *S. parauberis* strain investigated and the four reference strains of both species degraded esculin. Devriese et al. (1999) also used columbia sheep blood agar as well as esculin agar with columbia agar base, 0.1% esculin, 0.01% ferric citrate and 5%

sheep blood for cultivation, preliminary identification and differentiation of genus *Streptococcus* and *Enterococcus*.

In further studies the isolated esculin positive bacteria of the present investigation were differentiated in esculin degrading streptococci and enterococci. Enterococci have a natural resistance against bile, degrade esculin (Facklam and Moody 1970) and have the ability to grow in the presence of sodium azide (Reuter 1992). Different authors recommended various specific growth media for the isolation of enterococci. This included Slanetz and Bartley agar (Butaye et al. 1999; Devriese et al. 1999), esculin bile azide agar (Reuter 1978; Reuter 1992; Manero and Blanch 1999), columbia esculin blood agar with colistin and nalidixic acid (Butaye et al. 1999), streptococcus selective media, thallium acetate media and crystal violet azide dextrose blood media (Reuter 1978), Kanamycin esculin azide (KAA) agar (Reuter 1978; Reuter 1992; Devriese et al. 1993; Devriese et al. 1995; Butaye et al. 1999) and Citrate azide tween carbonate agar (CATC) (Reuter 1968a; Reuter 1968b; Reuter 1978; Reuter 1992). Similarly, Ellerbroek et al. (2000) used CATC agar, Esculin bile agar and Slanetz and Bartley agar for the isolation of *Enterococcus* species. A weak growth of bacteria of genus *Streptococcus* or *Lactobacillus* on CATC agar was reported by these authors. To further investigate the esculin positive bacteria of the present study, five different media specific for enterococci were used. This included CATC agar, KAA agar, Chromocult enterococci agar, Esculin bile agar and Slanetz-Bartley agar. None of the isolates of the present investigation grew on CATC and KAA media, respectively, while seven, nine and four isolates showed a weak growth on Chromocult enterococci agar, Esculin bile agar and Slanetz-Bartley agar, respectively. These growth patterns were clearly different to comparatively cultivated enterococci indicating that all five media could be used to differentiate between esculin degrading enterococci and *S. uberis*.

On the basis of the above mentioned criteria and the following results, 130 cultures investigated in the present study were preliminarily identified as *S. uberis* and one culture as *S. parauberis*. The following investigations included carbohydrate fermentation tests for arabinose, fructose, inulin, lactose, maltose, mannitol, raffinose,

ribose, saccharose, salicin, sorbitol, and trehalose; the determination of arginine hydrolysis and the determination of the enzymes β -D-glucuronidase, pyrrolidonyl aminopeptidase and hyaluronidase. The arginine hydrolysis, β -D-glucuronidase and pyrrolidonyl aminopeptidase enzyme activities were investigated with diagnostic tablets as substrates and the enzyme hyaluronidase with a plate test by cultivation of the isolates in close proximity of a mucoid growing *Streptococcus equi* subsp. *zooepidemicus* strain. All 130 strains preliminarily identified as *S. uberis* and both *S. uberis* reference strains fermented fructose, lactose, maltose, mannitol, ribose, saccharose, salicin, sorbitol, trehalose and hydrolyzed arginine, esculin and sodium hippurate. In addition 90 strains were positive for inulin fermentation, while all the *S. uberis* strains were unable to ferment raffinose and arabinose. Identical results were observed for the one *S. parauberis* investigated in the present study and both *S. parauberis* reference strains. All three *S. parauberis* strains fermented the carbohydrates mentioned above for *S. uberis* and hydrolyzed arginine, esculin and sodium hippurate. No fermentation could be observed for arabinose, inulin and raffinose. Seelemann (1954), Hughes (1960) and Obiger and Seelemann (1961) reported about similar fermentation and hydrolysis properties of *S. uberis*. According to Little et al. (1946) *S. uberis* fermented lactose, salicin and sorbitol. Similar fermentation reactions were also observed by Cullen et al. (1966, 1967 and 1969) and Sharma (1969). Roguinsky (1969, 1971) and Bridge and Sneath (1983) reported that all *S. uberis* strains investigated fermented inulin but not raffinose. In an additional study carried out by Roguinsky (1977) for the differentiation of *S. uberis* and *S. infrequens*, the *S. uberis* strains investigated fermented inulin and hydrolyzed sodium hippurate.

On the basis of these biochemical properties and corresponding to the cultural and biochemical criteria documented by various authors (Bramley 1980; Hahn 1980; Schaeren et al. 1984; Lerondelle 1985; Doane et al. 1987; Christ et al. 1988 and Lämmler 1991), all 130 isolates described in the present study could be classified as *S. uberis*.

Comparable to Garvie and Bramley (1979), William and Collins (1990) and Doménech et al. (1996), the 132 *S. uberis* and all three *S. parauberis* strains of the present investigation exhibited almost identical fermentation and hydrolysis reactions.

An analysis of the enzymes β -D-glucuronidase, pyrrolidonyl aminopeptidase, and hyaluronidase was determined to further differentiate *S. uberis* and *S. parauberis*. All 130 *S. uberis* isolates, including two *S. uberis* reference strains, were β -D-glucuronidase positive, while 116 *S. uberis*, also including both reference strains, exhibited pyrrolidonyl aminopeptidase enzyme activity and 47 *S. uberis*, including one *S. uberis* reference strain, a positive hyaluronidase test. All three *S. parauberis* isolates were pyrrolidonyl aminopeptidase positive whereas none of the isolates showed a β -D-glucuronidase and a hyaluronidase reaction. The present results corresponded to the investigations carried out for *S. uberis* reported by Bridge and Sneath (1983), Schaufuss et al. (1986), Christ et al. (1988), Schaufuss et al. (1989a) and Mathews et al. (1991b). In addition, Williams and Collins (1991) and Doménech et al. (1996) investigated *S. parauberis* species and reported about similar findings. Corresponding to these and the present results the enzyme β -D-glucuronidase seems to be the only criterion allowing a differentiation of *S. uberis* and *S. parauberis*.

In additional studies the 132 *S. uberis* and the three *S. parauberis* strains were investigated serologically. For this nine different group specific antisera were used. This included group A, B, C, E, G, L, P, U and V specific antisera. In studies of Little (1940) and Moreia-Jacob (1947), *S. uberis* strains occasionally reacted with group E specific antiserum. Later Sweeney (1964), Cullen (1968) and Sharma (1969) described that 17%-20% of the investigated *S. uberis* strains reacted with group E specific antiserum. Cullen (1969) also confirmed that some *S. uberis* strains reacted with group E specific antiserum. The number could be increased by formamide extraction of the antigen. The author suggested that at least three and probably 11 or more serological groups of *S. uberis* occur and serological cross reactions between groups are common.

Corresponding to the observations of these authors, the serological investigations of the present study revealed that 42 *S. uberis* cultures, also including

one *S. uberis* and one *S. parauberis* reference strain, reacted with group E specific antiserum, whereas 11 and 9 *S. uberis* strains reacted with group P and group U specific antiserum, respectively. One of the *S. uberis* isolates reacted with group A, two *S. uberis* strains simultaneously with group E and group P and one *S. uberis* with group E and group U specific antisera, respectively. One *S. uberis* reference strain reacted with group G specific antiserum. Comparable to the present and previous studies, Roguinsky (1969, 1971) also reported that *S. uberis* strains could serologically be classified into group E, P, G and U. Some of the strains investigated by Roguinsky (1969, 1971) simultaneously reacted with group E and group P, group P and group U, group P and group G specific antiserum, respectively; some strains were serologically non-groupable. A reaction of some *S. uberis* with group P, G, U and B specific antisera had also been reported by other authors (Schuman et al. 1972a; Schuman et al. 1972b; Garvie and Bramley 1979; Hahn 1981 and Christ et al. 1988). Lämmler (1991) also described that *S. uberis* may react with group E, G, P and U specific antisera alone or in combination.

Among the three *S. parauberis* strains one reference strain reacted with group E specific antiserum whereas the remaining two strains were non-groupable. The present results are in agreement with those of Garvie and Bramley (1979) and Williams and Collins (1990), who defined the species *S. parauberis* serologically. According to these authors, *S. parauberis* strains might exhibit a reaction with group E specific antiserum or remained non-groupable and appeared to be similar to *S. uberis*.

For further phenotypic characterizations lectin agglutination reactions were conducted with the lectins from *Helix pomatia* and *Dolichos biflorus*. In the present investigation 43 of the 132 *S. uberis* cultures agglutinated in the presence of the lectin from *Helix pomatia*. However, two strains self-agglutinated even though all strains were pretreated with trypsin. The three *S. parauberis* strains did not show any lectin reaction. In addition, all *S. uberis* showed no agglutination reaction with the lectin from *Dolichos biflorus*. Corresponding to the present results, Niewerth et al. (1987), Lämmler (1991), Christ and Lämmler (1992) and Abdulmawjood et al. (1996) demonstrated that some *S. uberis* specifically reacted with the lectin from *Helix*

pomatia but not with the lectin from *Dolichos biflorus*. In the studies of Schaufuss et al. (1986) the authors used the lectin of *Dolichos biflorus* as criterion to differentiate streptococci isolated from bovine mastitis. An agglutination reaction with the lectin of *Dolichos biflorus* was also used by Köhler und Prokop (1967), Niewerth et al. (1987), Nagai et al. (1994) as well as Abdulmawjood (1999) to identify streptococci of serological group C. The agglutination reaction was caused by a specific interaction of the N-acetyl-D-galactosamine specific lectin of *Dolichos biflorus* with the polysaccharide antigen of group C streptococci which has N-acetyl-D-galactosamine as major residue. The specific carbohydrate structure of the cell wall of *S. uberis* seemed to contain β -anomers of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. The *Helix pomatia* lectin has a specificity for α - and β configuration, whereas the lectin of *Dolichos biflorus* has a specificity for α -anomer configuration. Therefore the lectin from *Helix pomatia* but not from *Dolichos biflorus* could be used for characterization of *S. uberis*. Reactions with the lectin of *Helix pomatia* was also described for some cultures of *S. milleri* and *S. sanguis*, reactions with the lectin of *Maackia amurensis* for group B and with the lectins of *Dolichos biflorus* and *Wisteria floribunda* for group C streptococci (Kellens et al. 1993 and Nagai et al. 1994). The application of lectins in the field of microbiological diagnostics was summarized by Niewerth (1987) and Slifkin and Doyle (1990).

The *S. uberis* and *S. parauberis* cultures investigated in the present study were additionally examined for the presence of hemagglutinins. The hemagglutination reaction was carried out on glass slides with washed rabbit erythrocytes and hemagglutination positive and negative group B streptococci described by Wibawan et al. (1993) as control. However, none of the *S. uberis* and *S. parauberis* strains exhibited a positive hemagglutination reaction. Comparable to the present investigations, Soedarmanto (1996) studied group G streptococci isolated from cows, dogs and humans and obtained similar findings. None of the strains investigated by Soedarmanto (1996) showed any hemagglutination reactions. In contrast to the present findings, Abdulmawjood (1999) reported about positive (59%) as well as negative (41%) hemagglutination reactions among 39 *S. equi* subsp. *zooepidemicus* strains

investigated. A surface protein leading to hemagglutination of streptococci of serological group B seemed to have a significance for the adherence of the bacteria (Wibawan et al. 1993). The significance of hemagglutination reactions of *S. aureus* for the adhesion of the bacteria was described by Lindahl et al. (1989) and (1990).

A further phenotypic characterization was conducted by investigating the growth patterns of the *S. uberis* and *S. parauberis* strains of the present investigation in fluid media and soft agar. The cultivation of the *S. uberis* and *S. parauberis* strains in fluid media appeared in two different patterns. A uniformly turbid growth could be observed for 123 *S. uberis*, two *S. uberis* reference strains and one *S. parauberis* strain, respectively, while seven *S. uberis* strains and two *S. parauberis* reference strains grew as granular sediment with clear supernatant. In soft agar the *S. uberis* and *S. parauberis* strains appeared in three different growth patterns. A diffuse colony morphology could be observed for 101 of the *S. uberis* strains, one *S. parauberis* strain and one *S. uberis* and *S. parauberis* reference strain, respectively. A compact colony morphology could be observed for 18 *S. uberis* and one *S. uberis* and *S. parauberis* reference strain, respectively. A compact colony morphology together with a diffuse colony morphology was developed by 11 of the *S. uberis* strains. Comparable investigations have already been described for streptococci of serological group B and group L (Schaufuss et al. 1986; Lämmle and Blobel 1987b; Wibawan and Lämmle 1990b; Sippel 1994).

Comparing the relationship of the growth patterns in both cultivation media indicated that strains with uniformly turbid growth in fluid media generally formed diffuse colonies in soft agar, those with growth as sediment with clear supernatant generally formed compact colonies. However, some cultures did not show this relation.

According to Yoshida and Takeuchi (1970) as well as Yoshida et al. (1974), a diffuse growth of *S. aureus* in soft agar was related to an increased encapsulation of the bacteria. A comparable relation between growth properties in fluid media and soft agar, as described for the *S. uberis* isolates of the present investigation, could be observed for streptococci of serological group B (Wibawan 1993). Moreover,

Wibawan and Lämmle (1990b), Wibawan and Lämmle (1991), Wibawan et al. (1992) and Wibawan (1993) demonstrated a relationship between surface charge, chain length and growth characteristics in fluid medium and soft agar as well as the adhesion ability of the group B streptococci to epithelial cells. In addition a relation between growth characteristics in fluid medium and soft agar and the bacterial surface hydrophobicity could be demonstrated for streptococci of serological group B with and without polysaccharide and protein antigens. The soft agar technique was additionally used for demonstration of phase variation among streptococci of serological group B and *S. suis* (Wibawan and Lämmle 1990a; Salasia et al. 1994). Abdulmawjood (1999) and Wibawan et al. (1999) used the soft agar and other techniques for the isolation of phase variants of *S. equi* subsp. *zooepidemicus* and for characterization of mucoid and non-mucoid *S. equi* subsp. *zooepidemicus*.

According to the present results most of the isolates appeared with a uniformly turbid growth in fluid media and formed diffuse colonies in soft agar indicating a generally hydrophilic surface of the isolates possibly caused by a hitherto unknown microencapsulation of the bacteria.

Differences in surface hydrophobicity of the *S. uberis* and *S. parauberis* isolates of the present study could also be demonstrated by salt aggregation tests. Johnson and Wadström (1984) used the salt aggregation test to determine specific surface hydrophobicities among *S. aureus* strains. Among the strains investigated 51 of the *S. uberis* and one *S. parauberis* reference strain showed a specific aggregation reaction with ammonium sulfate, while 70 *S. uberis* strains, two *S. uberis* and one *S. parauberis* reference strain, respectively, did not show any reaction. A negative salt aggregation test for most of the isolates investigated again indicated a more hydrophilic surface of bacteria of this species. These results also corresponded to the findings mentioned above.

The antibiotic sensitivities of the *S. uberis* and *S. parauberis* strains of the present study were investigated with antibiotic discs on Mueller-Hinton blood agar. The results indicated that the *S. uberis* strains were highly susceptible to penicillin-G, cefotaxim, erythromycin, cefacetril and clindamycin and more than 90% of the

S. uberis strains were resistant to colistin. Some *S. uberis* strains displayed an intermediate susceptibility to gentamicin, minocyclin, tetracycline and sulphamethoxazole/trimethoprim, respectively. These results are generally in agreement with the findings of Cullen et al. (1969), King (1981), Bramley (1982), Doane et al. (1987), Bridge and Sneath (1983), Brown and Scasserra (1990) and Phuektes et al. (2001b).

The one *S. parauberis* strain and both *S. parauberis* reference strains displayed a sensitivity to penicillin-G, cefotaxim, colistin, minocyclin and tetracycline and were intermediately susceptible to cefacetril, clindamycin, erythromycin, gentamicin, and sulphamethoxazole/trimethoprim. This antibiotic sensitivity pattern mainly corresponded to the patterns reported by Doménech et al. (1996).

According to their biochemical, serological and other phenotypic characteristics the *S. uberis* and *S. parauberis* isolates of the present study appeared to be almost indistinguishable.

However, comparable to Jayarao et al. (1991), Jayarao et al. (1992) and Lämmler et al. (1998a) a molecular identification of both species could be performed by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene. Comparable to Jayarao et al. (1992) a RFLP analysis of the 16S rRNA gene of the *S. uberis* and *S. parauberis* isolates of the present investigation was performed using the restriction endonucleases *RsaI*, *AvaII* and *MspI*, respectively. Using the restriction enzyme *RsaI* the restriction pattern of *S. uberis* allowed a differentiation from *S. parauberis* by the absence of a 380 bp fragment for *S. uberis* which was present for *S. parauberis* and the presence of a 220 bp fragment for *S. uberis* which was absent for *S. parauberis*. Using the endonuclease *AvaII*, three fragments of 230, 310 and 900 bp could be observed for *S. uberis* whereas only two restriction fragments with a size of 230 and 1,200 bp could be observed for *S. parauberis*. Corresponding to Jayarao et al. (1992) as well as Lämmler et al. (1998a) all *S. uberis* and *S. parauberis* strains of the present investigation showed a specific restriction profile using the restriction enzymes *RsaI* and *AvaII*. However, in comparison to the latter the *MspI* restriction pattern showed similar fragment sizes of 100, 140, 170, 310 and 570 bp for all

investigated *S. uberis* and *S. parauberis* strains indicating that *RsaI* and *AvaII* but not *MspI* could be used for the differentiation of both species. A RFLP analysis of the 16S rRNA gene has also been used for characterization of *S. agalactiae* and *S. porcinus* (Lämmle et al. 1998b; Abdulmawjood et al. 1998). Comparable to the present results, these authors also found no intraspecies variations for the 16S rRNA genes of *S. agalactiae* and the serologically heterogeneous species *S. porcinus*. However, an intraspecies variation in the sequence of the 16S rRNA gene was observed for *S. suis* (Chatellier et al. 1998) and for *S. equi* subsp. *zooepidemicus* (Abdulmawjood and Lämmle 2000). Corresponding to the findings of Jayarao et al. (1991) and Williams and Collins (1991), the results of the present studies indicated that RFLP analysis could be used to differentiate *S. uberis* and *S. parauberis*.

In addition, a PCR-based identification with specific oligonucleotide primers targeted to species-specific regions of the gene encoding the 16S rRNA, the 16-23S rDNA intergenic spacer region and the gene encoding the 23S rRNA of *S. uberis* and *S. parauberis* was performed. Studying the 130 *S. uberis*, the one *S. parauberis* strain and the four reference strains of both species, a species-specific amplicons with sizes of 440 bp, 340 bp and 450 bp could be observed for *S. uberis* and species-specific amplicons with sizes of 880 bp, 200 bp, and 480 bp for *S. parauberis*. The amplified target genes did not show any size or sequence variations in the primer position. Comparable investigations were carried out by Forsman et al. (1997) investigating *S. uberis* specific parts of the 16-23S rDNA intergenic spacer region and by Hassan et al. (2001) using *S. uberis* and *S. parauberis* specific regions of the genes encoding the 16S rRNA and the 23S rRNA, and *S. parauberis* specific regions of the 16S-23S rDNA intergenic spacer region. In addition, Tilsala-Timisjärvi et al. (2000) used species-specific oligonucleotide primers targeted to the 16-23S rDNA intergenic spacer region for the differentiation of *S. uberis* and other pathogenic streptococcal and staphylococcal species. Moreover, Phuektes et al. (2001a) used a 16-23S rDNA intergenic spacer region based multiplex PCR assay for the identification and differentiation of *S. uberis* and other mastitis pathogens. Similarly, Riffon et al. (2001) described species-specific parts of the 23S rRNA gene and the 16-23S rDNA

intergenic spacer region of *S. uberis* as well as species-specific parts of the 23S rRNA gene of *S. parauberis*.

These and the present results indicated that the described gene sequences were unique for both species and that the use of species-specific oligonucleotide primer allowed a rapid and reliable identification and differentiation of *S. uberis* and *S. parauberis*.

An additional potential virulence factor investigated in the present study was the CAMP factor and the CAMP factor encoding gene *cfu*. The importance of “uberis factor” for the virulence of *S. uberis* has been pointed out by Skalka and Smola (1981). These authors parenterally administered an “uberis factor” containing exosubstance of *S. uberis* to rabbits and white mice causing the death of animals. In 1979, Skalka et al. reported that 35% of the *S. uberis* strains produced a hemolytically active exosubstance showing an identical effect as the CAMP factor of *S. agalactiae*. Similarly, Christ et al. (1988) and Lämmler (1991) found 10% and 28% CAMP positive *S. uberis* strains, respectively. In agreement with these studies five *S. uberis* strains of the present investigation exhibited a CAMP-like synergistic hemolytic activity on sheep blood agar. The remaining *S. uberis* as well as the *S. parauberis* strains did not show a comparable reaction. The results of the present study together with the previous findings indicated that the presence of CAMP factor in *S. uberis* seems to be rare. Corresponding to these results a CAMP like synergistic hemolysis of streptococci of serological group G in the presence of a β -hemolytic *S. aureus* strain has been described by Lämmler et al. (1987) and Soedarmanto and Lämmler (1996).

An amplification of the gene *cfu* encoding the CAMP factor of *S. uberis* was performed by Hassan et al. (2000) with phenotypically CAMP positive *S. uberis*. A comparable amplification of the CAMP factor encoding gene *cfu* of phenotypically CAMP positive *S. uberis* strains was performed in the present study using two different primer pairs. With these two primer pairs all five phenotypically CAMP positive *S. uberis* strains displayed an amplicon with a size of 870 bp and 680 bp, respectively. The amplicons had the expected sizes indicating no size polymorphisms. All phenotypically CAMP negative control strains of *S. uberis* and *S. parauberis*

investigated did not show a comparable amplicon. According to Jiang et al. (1996) the sequence of the CAMP factor gene *cfu* of *S. uberis* and the deduced amino acid sequence appeared to be highly homologous to the *cfb* gene and amino acid sequence of *S. agalactiae*. Similarly, Gase et al. (1999) described a sequence homology of CAMP factor gene *cfa* of group A streptococci, *cfb* of group B streptococci and *cfu* of *S. uberis*. These authors also suggested that CAMP factor and CAMP factor-like genes are fairly widespread among streptococci, at least in serogroups A, B, C, G, M, P, R and U. In addition, Hassan et al. (2000) found a close relation of the CAMP gene *cfa* of *S. pyogenes*, *cfb* of *S. agalactiae*, *cfu* of *S. uberis* and *cfg* of *S. canis*.

An additional potential virulence gene investigated in the present study was the gene *pauA/skc* encoding a plasminogen activator. According to previous investigations, bovine plasminogen activated by streptokinase seemed to be a virulence factor of *S. uberis* during early stages of infection. This activation might cause a rapid growth of the bacteria in the lactating bovine mammary gland (Leigh 1993). However, according to Leigh (1994) this plasminogen activator of *S. uberis* seems to be different from other bacterial proteins, which are capable of activating mammalian plasminogen. Schroeder et al. (1999) described that streptokinase plays an important role for the activation of plasminogen in group C streptococci grown in human and equine plasma. In addition, Caballero et al. (1999) elaborated that streptokinases secreted by non-human isolates of group C streptococci bound to different mammalian plasminogens and activated plasminogen, which could be recovered from the host from which the isolate had been obtained. These proteins belonged to a family of plasminogen activators.

For *S. uberis* the plasminogen activator gene *pauA* and the plasminogen activator gene designated as streptokinase gene *skc* was cloned and sequenced by Rosey et al. (1999) and Johnsen et al. (1999), respectively. According to Leigh (1994) the gene *pauA* was produced by the majority of the *S. uberis* strains isolated from clinical cases of bovine mastitis. It appeared to be the major plasminogen activator produced by *S. uberis*. Rosey et al. (1999) manifested that the *pauA* gene investigated from strains collected from two different geographical domains showed 99% identity.

These authors additionally studied 10 strains from the UK, USA and Denmark showing similar levels of homology in nine *S. uberis*. One *S. uberis* strain did not contain the *pauA* gene similarly to the reference strains used as control. The authors suspected that this specific plasminogen activator is highly conserved between distinct strains over a wide geographical area. Johnsen et al. (2000) also investigated plasminogen activation mediated by a novel two domain (α,β) streptokinase isolated from *S. uberis*. This streptokinase showed a specificity towards bovine plasminogen. According to these authors the plasminogen activator from *S. uberis* seemed to be related to the other known streptokinases.

Corresponding to the latter studies, the 132 *S. uberis* and three *S. parauberis* strains of the present investigation were further characterized for the presence of the genes *pauA/skc*. Using two different primer pairs, a *pauA/skc* gene with sizes of 1130 and 800 bp could be amplified for 128 of the *S. uberis*. Four *S. uberis* and three *S. parauberis* strains appeared to be negative. The amplicons had the expected sizes indicating no size polymorphisms. To determine the degree of homology, the nucleotide sequence of *pauA* described by Rosey et al. (1999) and *skc* described by Johnsen et al. (1999) were aligned. This alignment performed in the present study showed a high degree of homology between both nucleotide sequences. These alignment results explain that *pauA* and *skc* could simultaneously be amplified for an identical number of cultures. The present results confirmed that *pauA/skc* gene seems to be highly conserved among isolates of *S. uberis*. In a more recent study Ward and Leigh (2002) described that the *S. uberis* strains negative for *pauA* gene might possess a gene distinct from *pauA*. This gene *pauB* encoded a plasminogen activator with a molecular weight of 30 kDa. However, the frequency of this gene among *S. uberis* appeared to be low.

Earlier characterizations suggested that *pauA* from *S. uberis* was distinct from streptokinase nucleotide sequences of other bacteria by virtue of its molecular size and substrate specificity (Leigh 1993; Leigh 1994). However, Rosey et al. (1999) demonstrated a weak homology of *pauA* to streptokinase nucleotide sequences of other bacterial species. These authors speculated that an activation of bovine

plasminogen by the *pauA* gene product probably has arisen as a result of an independent evolutionary event, which was distinct from acquisition of streptokinase and streptokinase-like molecules. The genes of comparable plasminogen activators or streptokinases were isolated, cloned and sequenced for *S. pyogenes*, *S. equisimilis* and group G streptococci by Malke et al. (1985), Walter et al. (1989a) and Walter et al. (1989b), respectively. Malke (1993) demonstrated that the streptokinases of streptococci of serological groups A, C, and G are a group of extracellular proteins with a molecular mass of approximately 47 kDa. The nucleotide sequence alignment of group A, C, and G streptococci displayed 80 and 98% identity. Similar findings had been described by Mechold et al. (1993) and Frank et al. (1995). Recently, Johnsen et al. (1999) investigated a gene probe based on the streptokinase gene *skc* from *S. equisimilis*. The gene probe failed to hybridize with chromosomal DNA from three *S. uberis* strains. Moreover, Nowicki et al. (1994) cloned and sequenced the nucleotide sequence of plasminogen activator gene *esk* of *S. equisimilis*. According to Rosey et al. (1999), the nucleotide sequence of *pauA* showed no significant homology to the 5'-terminal nucleotide sequence of *esk*. These authors also proclaimed that the location of *pauA* within the chromosome of *S. uberis* was clearly different from that of the streptokinase genes *ska*, *skc*, *skg* in serogroup A, C and G streptococci.

To determine the epidemiological relationship of the collected *S. uberis* strains, a macrorestriction analysis of their chromosomal DNA was performed by pulsed-field gel electrophoresis (PFGE). The isolates were collected from bovine milk of a defined area within a time period of three months. This collection corresponded to the criteria of epidemiological isolates proposed by Tenover et al. (1995). These authors additionally defined a set of guidelines for interpreting DNA restriction patterns generated by PFGE and for using these results as epidemiologically useful information. Gordillo et al. (1993) used PFGE for typing group B streptococci and described that PFGE patterns could easily be discerned, interpreted and potentially utilized for epidemiological investigations.

In the present investigation the PFGE restriction patterns obtained from 69 selected *S. uberis* strains were comparatively investigated after digestion with the

endonuclease *Sma*I. This produced 8-13 distinct fragments and allowed a differentiation of the strains of the investigated herds. The results of the present study revealed nonidentical as well as identical PFGE patterns for isolates within different quarters of an individual cow and different cows within the same farm. Among 69 *S. uberis* strains isolated from 57 cows from 26 different farms 55 different DNA restriction patterns were observed, indicating that a wide variety of *S. uberis* strains might infect and cause mammary gland infection due to the contamination of the gland from the environment. This high degree of heterogeneity supports epidemiological studies by Baseggio et al. (1997), also suggesting a limited transmission of infection from cow-to-cow during milking process. These authors examined and differentiated *S. uberis*, *S. agalactiae* and *S. dysgalactiae* isolates by PFGE also after digestion with the restriction enzyme *Sma*I. The *S. uberis* isolates investigated in these studies displayed diverse restriction patterns. However, the investigated *S. dysgalactiae* had most diverse and complex restriction patterns. In contrast to the latter the species *S. agalactiae* had identical restriction patterns within the herds but distinct between herds. The studies of Douglas et al. (2000) additionally supported the results of the macrorestriction analysis of the *S. uberis* isolates of the present investigation. According to these authors 330 different PFGE patterns could be observed from 343 isolates with a degree of similarity of 45%. In addition Wang et al. (1999) reported that *S. uberis* had most diverse PFGE patterns as compared to *S. agalactiae* and *S. dysgalactiae*. According to these authors, 74 distinct PFGE patterns could be observed among 130 *S. uberis* strains collected from 73 different cows of 3 farms. In contrast to *S. uberis*, the *S. agalactiae* isolates examined by Wang et al. (1999) exhibited, corresponding to the results of Baseggio et al. (1997), identical patterns within the same farm but different patterns between various farms. The latter indicated that a single clone was transmitted between cows. Fink et al. (2000) also analyzed and compared macrorestriction patterns of *S. agalactiae* isolated from bovine mastitis. According to these authors, also a single clone seemed to be responsible for the mastitis situation within a herd. The clones differed between herds. Moreover, Annemüller et al. (1999) analyzed PFGE patterns of *Staphylococcus aureus* strains

isolated from cows with mastitis. These studies revealed that isolates from a single farm generally had identical restriction patterns. This could also be observed for isolates of different herds. Akineden et al. (2000) also described that *S. aureus* had identical PFGE restriction patterns within the same farm but different patterns between the farms investigated.

Despite the degree of heterogeneity in DNA restriction pattern, some *S. uberis* strains of the present study isolated from a single cow as well as from different cows of the same farm displayed identical PFGE patterns, indicating that some *S. uberis* strains might be transmitted from quarter to quarter and cow to cow of a single farm. This also corresponded to the findings of Phuektes et al. (2001b). These authors investigated the epidemiological status of *S. uberis* mastitis in dairy cows and detected nonidentical and also identical PFGE patterns. Identical patterns could be observed for nine of 38 *S. uberis* isolated from different quarters of an individual cow and from cows within the same herd.

According to the results of the present study, a macrorestriction analysis of the *S. uberis* isolates by PFGE appears to be a useful and reliable method to study the epidemiological relationship of the investigated strains.

The present study was performed to identify *S. uberis* and *S. parauberis*, isolated from bovine mastitis by conventional and molecular methods. The latter included RFLP analysis and the detection of species-specific target genes. The isolates were further characterized for potential virulence markers including the CAMP factor gene *cfu* and the plasminogen activator gene *pauA/skc* and for epidemiological relations by macrorestriction analysis of their chromosomal DNA. The described methods might be useful for identification and further characterization of *S. uberis* and *S. parauberis* and might help to determine the importance of both species in bovine mastitis.

6. Summary

In the present study 130 *S. uberis* strains and one *S. parauberis* strain isolated from bovine milk samples of 59 different farms of various locations in Hesse, Germany, were comparatively investigated together with four reference strains of both species for cultural, biochemical and serological properties. The *S. uberis* and *S. parauberis* strains generally did not grow on media specific for enterococci, whereas after cultivation on blood agar both species were α - or non-hemolytic. All *S. uberis* strains produced the enzyme β -D-glucuronidase, the *S. parauberis* were negative for this enzyme. Serogrouping revealed a positive reaction for 42 *S. uberis* strains and one *S. parauberis* reference strain with group E specific antiserum and for some strains with specific antisera of the serological groups P, U and A. However, most of the *S. uberis* and two *S. parauberis* strains were non-groupable. Some of the *S. uberis* strains agglutinated with the lectin of *Helix pomatia*, and showed a CAMP-like reaction of complete hemolysis in the zone of staphylococcal β -toxin. The *S. uberis* and *S. parauberis* strains displayed a generally hydrophilic surface. This could be demonstrated by determination of growth patterns in fluid media and soft agar and by salt aggregation tests. Antibiotic resistances could be observed for most of the strains for colistin, sulphamethoxazole/trimethoprim, tetracycline, clindamycin, erythromycin and gentamicin. All strains were sensitive to penicillin-G.

The *S. uberis* and *S. parauberis* isolates were further analyzed by molecular methods. This was performed by amplification of the gene encoding the *S. uberis* and *S. parauberis* 16S rRNA gene by polymerase chain reaction and subsequent digestion of the respective gene with the restriction enzymes *Rsa*I and *Ava*II. The species identity could additionally be confirmed by amplifying species-specific parts of the genes encoding the 16S rRNA, the 23S rRNA and of the 16S-23S rDNA intergenic spacer region. For CAMP positive *S. uberis* strains the gene *cfu* could be amplified using two different primer pairs. In addition the gene for the potential virulence factor streptokinase, the *skc/pauA* gene, could be amplified for 128 of the 132 *S. uberis* but not for *S. parauberis*.

To analyze the epidemiological relationship, arbitrarily selected isolates of *S. uberis* were subjected to DNA fingerprinting by macrorestriction analysis of their chromosomal DNA by pulsed-field gel electrophoresis. The DNA fingerprinting revealed that most of the isolates were not related to each other. However, identical DNA patterns were noted for some of the isolates within different quarters of an individual cow and also for different cows within the same farm. The generally not related DNA patterns indicated that *S. uberis* is a pathogen with multiple environmental habitats and that infections are caused by a great variety of strains.

The conventional and molecular methods used in the present study allowed a reliable identification and further characterization of *S. uberis* and *S. parauberis* and might help to investigate the importance of both species as causative agents of bovine mastitis.

7. Zusammenfassung

In den vorliegenden Untersuchungen wurden 130 *S. uberis*- und eine *S. parauberis*-Kultur, isoliert aus Kuhmilchproben von 59 unterschiedlichen Betrieben aus verschiedenen Regionen Hessens (Deutschland), sowie vier Referenzstämme beider Spezies vergleichend kulturell, biochemisch und serologisch untersucht. Sowohl die *S. uberis*- als auch die *S. parauberis*-Kulturen zeigten im allgemeinen kein Wachstum auf Enterokokken-Selektivnährmedium und wuchsen auf Schafblutagar α - oder nicht hämolysierend. Alle *S. uberis*-Kulturen produzierten das Enzym β -D-Glucuronidase, die *S. parauberis*-Kulturen waren β -D-Glucuronidase-negativ. Bei der serologischen Gruppenbestimmung zeigten 42 *S. uberis*-Kulturen und ein *S. parauberis*-Referenzstamm eine positive Reaktion mit dem spezifischen Antiserum der Gruppe E, sowie einige Kulturen mit Antiseren der Gruppen P, U und A. Die meisten der *S. uberis*- und zwei der *S. parauberis*-Kulturen konnten keiner Serogruppe zugeordnet werden. Einige der *S. uberis*-Kulturen agglutinierten mit dem Lektin von *Helix pomatia* und zeigten eine CAMP-ähnliche Reaktion mit vollständiger Hämolyse im Bereich des Staphylokokken- β -Toxins. Die *S. uberis*- und *S. parauberis*-Kulturen wiesen überwiegend eine hydrophile Oberfläche auf. Dies konnte anhand der Wachstumseigenschaften in Flüssigmedium und Soft-Agar sowie mittels Salz-Aggregationstests gezeigt werden. Antibiotische Resistenzen waren bei den meisten Kulturen für Colistin, Sulphamethoxazol/Trimethoprim, Tetracyclin, Clindamycin, Erythromycin und Gentamicin nachweisbar. Alle Kulturen erwiesen sich als empfindlich gegenüber Penicillin-G.

Die *S. uberis*- und *S. parauberis*-Isolate konnten im weiteren mit Hilfe molekularer Methoden charakterisiert werden. Dies erfolgte durch Amplifizierung des 16S rRNA-Gens durch Polymerasekettenreaktion und anschließendem Restriktionsverdau des Amplikons mit den Enzymen *RsaI* und *AvaII*. Die Identifizierung konnte im weiteren durch Amplifizierung speziesspezifischer Abschnitte der 16s rRNA- und 23S rRNA-Gene sowie der 16S-23S rDNA „intergenic spacer“-Region bestätigt werden. Die Amplifizierung des *cfu*-Gens der CAMP-positiven *S. uberis*-Kulturen erfolgte unter Verwendung von zwei

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unterschiedlichen Primerpaaren. Zusätzlich konnte das Gen für den potentiellen Virulenzfaktor Streptokinase, das *skc/pauA*-Gen, bei 128 der insgesamt 132 *S. uberis*- nicht jedoch bei den *S. parauberis*-Kulturen festgestellt werden.

Der Nachweis epidemiologischer Beziehungen ausgewählter *S. uberis*-Kulturen erfolgte durch Makrorestriktionsanalyse der chromosomalen DNA der Kulturen und anschließender Pulsfeldgelelektrophorese. Dieses „DNA-Fingerprinting“ ergab keine enge verwandschaftliche Beziehung zwischen den untersuchten Kulturen. Bei einigen Isolaten aus unterschiedlichen Vierteln einer Kuh, sowie bei Isolaten von verschiedenen Kühen eines Betriebes waren allerdings auch identische DNA-Muster feststellbar. Die überwiegend nicht übereinstimmenden DNA-Muster zeigten, dass *S. uberis* ein Mikroorganismus mit unterschiedlichsten Lebensräumen ist und die Infektionen in der Regel von einer Vielzahl verschiedener Stämme hervorgerufen werden.

Die in den vorliegenden Untersuchungen benutzten konventionellen und molekularen Methoden erlaubten eine Identifizierung und weitergehende Charakterisierung von *S. uberis* und *S. parauberis* und könnten Aufklärung über die Bedeutung beider Spezies als Verursacher boviner Mastitiden geben.

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