CHARACTERIZATION OF SLIME PRODUCTION AND PLASMID PROFILING OF THE COAGULASE-NEGATIVE STAPHYLOCOCCI

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CHARACTERIZATION OF SLIME PRODUCTION AND PLASMID PROFILING OF THE COAGULASE-NEGATIVE STAPHYLOCOCCI

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LIST OF ABBREVIATIONS

API	Analytical Profile Index
CAPD	Continuous Ambulatory Peritoneal Dialysis
CDC	Centres for Disease Control
dd water	Double Distilled water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
MIC	Minimum Inhibitory Concentration
NaAc	Sodium Acetate
PVC	Poly Vinyl Chloride
PVE	Prosthetic Valve Endocarditis
RF	Retardation Factor
SDS	Sodium Dodecyl Sulfate
SXT	Sulfamethoxazole/Trimethoprim Lactate
TES	TRIS-EDTA-Saline
TRIS	Tris(hydroxymethyl)aminomethane
TSB	Tryptic Soy Broth
UTI	Urinary Tract Infection
UV	Ultra Violet

ABSTRACT

Coagulase-negative staphylococci are a major component of the skin and mucous membrane flora. As a result of this, when these organisms were isolated from clinical specimens they were often discounted as being a contaminant or innocuous. Recently the coagulase-negative staphylococci have emerged as a significant cause of infection. These include major infections such as prosthetic valve endocarditis, urinary tract infections, and shunt, catheter and prosthesis infections.

Despite the fact that these organisms had been found to be a major cause of infection, little was understood about the epidemiology and pathogenesis of coagulase-negative staphylococci infections.

A typing technique such as plasmid profiling would be very useful in epidemiology studies of these organisms. Most published methods proved to be inadequate for our purposes. However, the CDC method, following a few alterations, proved to be a good, consistent plasmid profiling method.

Although little is known about the virulence factors of the coagulase-negative staphylococci, slime production had been associated with increased virulence. Curing, transformation, and plasmid analysis results indicated that the production of slime was mediated by chromosomal genes.

Experiments were also carried out on the slime, and slime producing organisms to develop a quick screen to differentiate between slime and nonslime producers. Biological staining with alcian blue and congo red was unable to differentiate these organisms. Attempts to raise antibodies against the slime also failed. It appeared that the slime, isolated from the slime producing organisms was a poor immunogen in a rabbit model.

INTRODUCTION

The human body is a major reservoir of the coagulase-negative staphylococci. These organisms prefer the skin and mucous membranes of the nasopharynx and other areas adjoining various body orifices (Kloos, 1982).

Historically these organisms were considered to be mere contaminants when isolated from clinical specimens due to their ubiquitos nature on the human body (Sewell, 1984). It is now known that the coagulase-negative staphylococci are responsible for numerous infections. A list of these infections, compiled by Sewell (1984), included major infections such as prosthetic valve endocarditis, urinary tract infections, shunt, and catheter infections.

Because these organisms were believed to be innocuous, little work was done on their taxonomy and virulence. Baird-Parker (1963) was the first researcher to develop a significant classification scheme for the Staphylococci. Since this time Baird-Parker (1974), Kloos and Schleifer (1975) and other researchers have come to recognize many more staphylococcus species. Today there are 11 recognized human species, 10 of which are coagulase-negative staphylococci (Doern, 1984). With the recent upsurge in molecular genetic techniques new methods became available to study and type organisms. One of these methods was plasmid profiling. This method has been used to analyze both gram negative and gram positive bacteria. Researchers such as Parisi and Hecht (1980), Archer et al (1982), Maki et al (1982), and Archer et al (1984) all have used this technique to investigate the coagulase-negative staphylococci and their plasmid content. We undertook to develop a plasmid profiling technique

which could consistently give reproduceable and accurate plasmid profiles for any coagulase-negative staphylococci organism studied.

Due to the fact that these organisms were always considered innocuous little was known about their virulence factors. In recent years the work on these virulence factors has increased. Solymossy (1982) Aldridge (1982), and Males (1975) have all looked at the virulence factors of these organisms. However most of the work on virulence factors was centered around slime production. The production of slime had been directly correlated with increased virulence by Toshida and Ichiman (1981) and Christensen (1983). Other researchers such as Sheth et al (1983), Peters et al (1982) and Gray et al (1984) looked at the adhesion and effects of slime production. We decided to study the slime of the coagulase-negative staphylococci by determining if the genes encoding for slime production were plasmid or chromosomal mediated. We proposed to do this with curing and transformation experiments. We also hoped to establish a quick screen to differentiate between slime positive and slime negative organisms. We proposed to do this using biological staining and antibodies, produced in rabbits, incorporated into agarose.

LITERATURE REVIEW

A. COAGULASE NEGATIVE STAPHYLOCOCCI

1. Taxonomy

The family Micrococcaceae consists of three genera, the Micrococci, the Staphylococci, and the Planococci. This family contains gram-positive cocci that are facultatively anaerobic and grow in irregular clusters. The staphylococci appear to be the only genus of medical importance in the family Micrococcaceae. The name staphylococcus originates from the Greek nouns, staphyle (a bunch of grapes) and coccus (a grain or berry). This name was introduced to describe the organisms as seen by early investigators in pus from surgical wounds (Zinsser et al, 1980).

In 1884 Rosenbach was the first to attempt to differentiate the genus staphylococcus into species. He did this on the basis of an obvious and simple characteristic: colony pigmentation. Rosenbach's results indicated the presence of two species in this genus, Staphylococcus aureus which produced a golden pigmentation, and Staphylococcus albus which produced a white pigmentation. In 1885, Passet added a third species, the lemon-colored Staphylococcus citreus, to this genus. However, this phenotypic characteristic was found to be geneticaly unstable in many strains, making it an unsatisfactory criterion to speciate organisms (Kloos, 1980).

Von Daranyi (1925) realized that the blood plasma clotting ability of the staphylococci could be of value in typing these organisms. Von Daranyi found that S. aureus could coagulate blood plasma, whereas the S. albus

could not coagulate blood plasma. Hence S. aureus was coagulase positive and S. albus was coagulase negative. However, this test did not gain acceptance until the 1930's when the correlation between the coagulase reaction and pathogenicity became apparent.

During the period of the 1940's and 50's little progress was made in clarifying the taxonomic position of these orgnisms. Hucker (1948) lumped the staphylococci and micrococci into a single genus; micrococcus, while Shaw (1951) placed the two into the single genus staphylococcus. Evans (1955) became dissatisfied with the lumping of staphylococci and micrococci into a single group and proposed separating the two on their ability to grow in the presence of oxygen. Species which could grow with or without oxygen (facultative species) became staphylococcus, while obligate aerobes became part of the genus micrococcus.

Silvestri and Hill (1965), and Auletta and Kennedy (1966) looked at the deoxyribonucleic acid base composition of the staphylococci and the micrococci. They found that the staphylococci had a guanine plus cytosine content of from 30-39%, whereas the micrococci had a guanine and cytosine content of between 63-73%. This was seen as convincing evidence that the staphylococci and micrococci were not as closely related as was believed. Other evidence supporting this belief arrived when the International Association of Microbiological Societies (1965) introduced a standard test for separating staphylococci from micrococci. It was based on the ability of staphylococci to grow and produce acid anaerobically from glucose in a peptone-yeast extract medium containing the pH indicator bromo-cresol purple. Thomas and Schulhardt (1964) supported this separation, based on the susceptibility of these organisms to lysostaphin. The anaerobic glucose fermenting staphylococci were attacked by the lysostaphin whereas

the micrococci were not attacked. It became established that there were two separate genera in the family Micrococcaceae, the Staphylococci and the Micrococci.

Baird-Parker (1963) was the first taxonomist to come up with a significant typing and classification system for the staphylococci. Baird-Parker looked at 1,250 gram positive, catalase-positive cocci isolated from bacon, pig and human skin, and dust. He compared their morphology, physiology and biochemical characteristics. His results were in agreement with previous work by Evans (1955) who found that the staphylococci and micrococci could be differentiated by the ability of staphylococci to grow and produce acid from glucose anaerobically. The classification that evolved from this study classified the staphylococci into six subgroups. Subgroup I was the species S. aureus as defined by Shaw et al (1951). The other five staphylococci species were found to be coagulase negative and did not produce acid from mannitol. The S. aureus species did produce acid from mannitol.

Baird-Parker (1965) made another survey of the Micrococcaceae using 607 cultures of gram-positive, and catalase positive cocci received from workers and collections in different parts of the world. His results indicated no change in the number of subgroups into which the staphylococci could be differentiated.

Baird-Parker (1974) published one more major classification scheme for the staphylococci. In this study he expanded the number of recognized staphylococcus species to nine. These species were S. aureus, S. epidermidis (4 biotypes) and S. saprophyticus (4 biotypes). Biotypes represent subspecies which differ in only a few biochemical tests.

Kloos and Schleifer (1975) published a simplified scheme for the identification of staphylococci species. This scheme recognized ten staphylococci species including S. aureus, plus nine separate coagulase negative species.

Since 1975 there have been more coagulase negative staphylococci species recognized. This has resulted from DNA hybridization studies amongst the species as well as further work on phenotypic characteristics (Kloos, 1980; Kloos and Schliefer, 1983). As a result of this and other work, a list of currently recognized species of staphylococci would include eleven human species of which ten are coagulase negative, and six non-human coagulase negative species. A list of these species, as published by Doern (1984), was as follows:

TABLE I: Currently Recognized Species of Staphylococci

Human Species	Nonhuman Species
S. aureus	S. intermedius
S. epidermidis	S. hyicus ssp hyicus
S. saprophyticus	S. hyicus ssp chromogens
S. haemolyticus	S. sciuri ssp sciuri
S. hominis	S. sciuri ssp lentes
S. warneri	S. caseolyticus
S. captitis	S. carrious
S. cohnii	S. simians
S. xylosus	
S. simulans	
S. auricularis	

2. Natural Habitat

The human body is a major reservoir for the coagulase-negative staphylococci. Kloos (1982) described their preferred natural habitat as the skin and mucous membranes of the nasopharynx and other areas adjoining varius body orifices.

Kloos and Schleifer (1980) found that the major resident species living on humans include S. epidermidis, S. hominis, S. capitis, S. haemolyticus and S. auricularis. Species such as S. warneri, S. saprophyticus, S. cohnii, S. xylosus, and S. simulans are usually found in small numbers or as transient populations on humans.

Kloos (1982) also reported that some species of staphylococci demonstrate a strong preference for certain niches within the human ecosystem. S. capitis is found only on the head, particularly the scalp and forehead where sebaceous glands are numerous and well-developed. S. auricularis is the major species of the outer ear. S. hominis and S. haemolyticus prefer areas of the skin where apocrine glands are numerous. S. epidermidis is found in the anterior nares, axillae, groin and perineal areas, the feet and forehead, as well as the face. S. aureus the coagulase positive species prefers the anterior nares.

Kloos and Musselwhite (1975) investigated the quantity of all bacterial species on human skin. Their results indicated that S. epidermidis is the most prevalent species of coagulase negative staphylococci residing on human skin.

3. Physical Characteristics

Baird-Parker (1972) described the genus staphylococcus as non motile, gram-positive, and catalase positive cocci 0.5-1.5 um in diameter. These

organisms have the ability to divide in more than one plane to form irregular clusters of cells. This is highly characteristic of the Staphylococci.

The cell wall of the staphylococci, like other gram positive bacteria, consists mainly of peptidoglycan and teichoic acids. The thickness of the cell wall will vary according to the particular strain, and the age of the culture. Suganuma (1966) showed that the walls of young cells are about 15nm thick, while the cell wall of older staphylococci cells will be approximately 80nm thick.

The structure of the staphylococci peptidoglycan was determined by Strominger and Ghuysen (1967) by studying the simple degradation products obtained by the action of hydrolytic enzymes. Teichoic acids, as shown by Archibald and Bradley (1966), are charged polymers in which repeating units containing ribitol or glycerol are held together by phosphodiester groups. Teichoic acids are responsible for much of the serological specificity of a cell and form part of the phage receptor sites as suggested by Archibald (1972). Davie and Brock (1966) found that in certain bacteria teichoic acids influence the action of lytic and autolytic enzymes on the cell. James and Brewer (1968) demonstrated that they also contribute to the surface charge of the cell.

Initial studies by Archibald (1972) demonstrated that the presence of ribitol teichoic acid was specific for coagulase positive staphylococci and that glycerol teichoic acid was specific for coagulase negative staphylococci. However work by Endl et al (1983) proved that this was not true. In their work they showed that some S. aureus strains did contain a glycerol teichoic acid, and that some coagulase negative staphylococci strains did contain a ribitol teichoic acid.

Other characteristics of the staphylococci, as reported by Baird-Parker (1972) were as follows. They are facultative anaerobes growing best in the presence of air. Aerobic growth requires a medium containing amino acids, and growth factors, while anaerobic growth requires all of these plus uracil and a fermentable carbon source. The staphylococci are mesophiles, growing between 6.5-46^{o}C, the optimum temperature is 35-40^{o}C. They will grow at pH values between 4.5 and 9.3, the optimum pH being between 7-7.5. Most strains can grow in 1.5% sodium chloride or 40% bile. They are sensitive to lysis by lysostaphin, which breaks the pentaglycine bridges in their peptidoglycan, but they are resistant to lysis by lysozyme. Their metabolism is both respiratory and fermentative. Finally their GC content ranges from 30-40%.

4. Clinical Significance

Historically the coagulase positive S. aureus were considered to possess pathogenic potential, whereas the coagulase negative staphylococci were considered to be avirulent organisms (Sewell, 1984). When a coagulase negative staphylococcus was isolated from a clinical specimen, it was almost always considered to be a contaminant due to its ubiquitous nature.

As stated by Christensen (1983), it was believed that the staphylococci act as saprophytes as part of the skin's normal flora. As saprophytes they limit the influx of virulent and other bacteria onto the various body surfaces. At the same time, the coagulase negative staphylococci were themselves held in check from further invasion by the host's dermal barrier and immunological system. When these defences were impaired, these usually saprophytic coagulase negative staphylococci could cause serious human disease (Gemmel, 1981; Sewell, 1982).

The infections caused by coagulase negative staphylococci include the following: Intravenous cannulae associated infections, subacute bacterial endocarditis, prosthetic valve endocarditis, urinary tract infections, peritonitis in patients on peritoneal dialysis, surgical wound infections, vascular graft infections, prosthetic joint infections, ocular infections, minor skin abscesses, and infections of the central nervous system in patients with ventricular drainage devices such as shunts (Sewell, 1984).

The role of Staphylococcus saprophyticus as an etiologic agent of urinary tract infections (UTI's) was not fully appreciated until quite recently. One reason is that coagulase negative staphylococci were generally considered apathogenic in the urinary tract. Another reason was that unless appropriate diagnostic and screening methods were used, the diagnosis of such infections was missed (Hovelius and Mardh, 1984).

Hovelius et al (1984) studied 95 Swedish women, 15-45 years old, presenting symptoms of distal UTI. They found S. saprophyticus to be the causative agent in 27% of the cases. Only E. coli, at 41%, caused more UTI'S. Hovelius et al (1984) also looked at UTI in males and found that S. saprophyticus was the most common pathogen in elderly patients. Other investigators Jordan et al (1980), and Wallmark et al (1978), have also shown S. saprophyticus to be an important pathogen in UTI.

Hovelius and Mardh (1984) looked for possible reservoirs of S. saprophyticus and determined that S. saprophyticus is not part of the normal flora of skin, but rather it appeared transiently and in low numbers. It has been isolated from the skin, the periurethra and urethra.

The coagulase negative staphylococci can also, under the right conditions, cause endocarditis in humans. Non-prosthetic valve or subacute endocarditis is rarely caused by coagulase negative staphylococci.

Estimates of non-prosthetic valve endocarditis, caused by coagulase negative staphylococci, range from 1-3% (Tuazon and Miller, 1983) to 2-6% (Lancet, 1981).

Although the coagulase negative staphylococci are rare pathogens in subacute endocarditis, they have become very common pathogens in acute or prosthetic valve endocarditis (PVE). Many researchers have looked at PVE, and have found coagulase negative staphylococci to be the most frequent causative organism. For instance, the Lancet (1981) reported that coagulase negative staphylococci were responsible for 37% of all PVE cases. Masur and Johnson (1980) in a PVE outbreak found the coagulase negative staphylococci to be responsible for 48% of their cases, while Dismukes et al (1973) found the coagulase negative staphylococci responsible for over one third of PVE cases.

Another example of an important infection caused by the coagulase-negative staphylococci is peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (CAPD). Doern (1984) reported that over one third of such infections were caused by coagulase negative staphylococci, while a study done by Rubin et al (1980) found the coagulase negative staphylococci to be responsible 31% of the time. Grefberg et al (1984) found the coagulase negative staphylococci to be the causative organism 57% of the time in 129 episodes.

Coagulase-negative staphylococci have also been implicated in various ocular infections. Mahajan et al (1980) state that S. epidermidis has been recognized as a pathogen in corneal ulcers, endophthalmitis, and blepharoconjunctivitis.

The coagulase negative staphylococci have also been found to be the causative organism in infections arising when a foreign body, such as a

shunt, a catheter or other prosthetic devices have been placed into the body.

Schoenbaum et al (1975) studied individuals over a ten year period, who had cerebrospinal fluid shunts and developed infections. The results of this study found the coagulase negative staphylococci to be responsible for one half of the infections.

More recently Togeu (1985) reported that the coagulase negative staphylococci are responsible for 60-75% of shunt infections. Togeu (1985) also speculated that these bacteria have special properties which allows them to bind to the shunts and become protected from the victim's immune system.

Examination of infected joint prosthesis demonstrates the importance of coagulase negative staphylococci in these infections. Lowy and Hammer (1983) looked at infection following total hip replacement, and found the coagulase negative staphylococci to be the causative organism in 20-50% of cases. Wilson (1977) also looked at total hip replacement infections and found the coagulase negative staphylococci to be responsible for 50% of their cases. Wilson et al (1973) looked at femoral replacements and resulting infections and found the coagulase negative staphylococci to be the pathogenic organism in 35% of cases.

Thus it can be said that the coagulase negative staphylococci, which at one time were considered to be saprophytic organisms, are, in fact, a major cause of bacterial infections. Perhaps Christensen (1983) said it best when he stated that coagulase negative staphylococci infections were a disease of medical progress, and as more and more medical devices are inserted into the human body, we are encountering more and more infections related to the use of these devices.

5. Virulence Factors of the Coagulase Negative Staphylococci

The fact that the coagulase negative staphylococci were never considered as pathogens, until recently, has meant that little is known about their virulence factors.

S. aureus has many virulence factors, some of which may also be virulence factors of the coagulase negative staphylococci. However this has not been demonstrated. These factors as described by Davis (1980) include three hemolysins (alpha, beta, and gamma), staphylokinase, Panton-Valentine leukocidin, and enterotoxins A-E.

Sewell (1984) and Lowy et al (1983) agree that a prerequisite for infection by coagulase negative staphylococci was a break in host defences. After this break in defence the coagulase negative staphylococci are able to cause an infection in the host. How infection occurs is not known, however some research has been done on the possible mechanisms, which has resulted in some published theories.

Kloos (1982) mentioned that Ryden et al are working on fibronectin binding in wound tissue colonization, and its possible role in coagulase negative staphylococci pathogenicity.

Solymossy et al (1982) discovered a cytotoxic material released from S. epidermidis which they labelled F_2. This substance had a molecular weight of 4×10^3, contained 9.5% protein, and 73% carbohydrate, and was released from S. epidermidis in phosphate buffered saline. This material was soluble in ethanol and was heat resistant. The biological characteristics of this material were that it blocked amino acid uptake and E-rosette formation of human tonsillar and blood lymphocytes. In mouse hepatocytes, this material inhibited protein synthesis in the presence of calcium ions. These results indicated that this material, released from S. epidermidis,

could damage eukaryotic cell membranes. The mechanism of action is not known, however it probably binds to membrane proteins responsible for rosette formation and amino acid uptake. Complete membrane disorganization could be excluded because glucose and thymidine transport across the membrane were not inhibited by F_2.

Aldridge (1982) reported that the coagulase negative staphylococci produced neither the coagulase nor the wide variety of extracellular enzymes formed by S. aureus. However, Gemmell and Roberts (1973) developed a colony overlay technique that utilized a semisolid agar overlaying a monolayer of skin fibroblasts to assay the toxigenicity of the coagulase negative staphylococci. They found the cytopathogenic strains were primarily S. epidermidis, S. saprophyticus, and S. haemolyticus. This study also demonstrated a good correlation between strains ability to produce exotoxins and their ability to cause infection. Some of the toxins and enzymes that coagulase negative staphylococci were found to produce were alpha and beta haemolysins, succinic oxidase factor, DNAase, phosphatase, gelatinase and lipase. Males (1975) looked at 228 clinical isolates of coagulase negative staphylococci and found that 81.8% of these produced a haemolysin. Males also found that many of his isolates produced DNAase, urease, gelatinase, caseinase, and lysozyme. Gemmel and Thelestam (1981) did a study on 50 clinical isolates of coagulase negative staphylococci. They looked at lysis of human erythrocytes, leakage of a radioactive marker from human embryonic lung fibroblasts by culture filtrates, and direct cytotoxicity of growing bacteria towards mouse skin fibroblasts in an agar overlay assay. Their results indicated that coagulase negative staphylococci isolated from a variety of clinical conditions could produce a potential haemolysin, capable of enhancing their pathogenicity for man.

Hovelius and Mardh (1984) looked at pathogenic factors of S. saprophyticus and concluded that these factors were poorly defined. the S. saprophyticus does not produce a DNAase, phosphatase or any endonucleases; factors which have been associated with pathogenicity of S. aureus. However, they did find that S. saprophyticus showed a tropism for the epithelial lining of the urinary tract, the only organ system in which this organism is known to cause disease. In vitro tests have shown that the bacterium has a selective ability to adhere to urothelium, so specific adherence was important to pathogenicity in S. saprophyticus.

Another posulated pathogenic factor of the coagulase negative staphylococci was slime production. Although little research has been done on slime production, enough has been done to indicate that there is a direct correlation between slime production and pathogenicity. Christensen (1983) examined strains of coagulase negative staphylococci from patients with intravascular catheter-associated sepsis, and found that the coagulase negative staphylococci strains that produced slime were more pathogenic than the strains that did not produce slime.

In nature, bacteria may have a mass of tangled polysaccharide fibers extending from the bacterial surface. This mass of fibers is known as the glycocalyx or slime, and it may mediate adhesion of bacteria in microcolonies to various surfaces, both artificial and natural.

Bayston and Penny (1972) made the first correlation between slime production and pathogenicity. They looked at slime production on cerebrospinal shunts, and found that the slime protects bacterial cells from the action of lysozyme. They also speculate that slime may play a part in inhibiting the action of antibiotics against the infecting organism. Lowy et al (1983) also found that slime enhances bacterial

adhesion to catheters in vitro and these attached colonies were extremely resistant to antiseptic agents and biocides. Christensen et al (1982) looked at coagulase-negative staphylococci adherence to smooth surfaces and found that 63% of clinical isolates produced slime, whereas only 37% of randomly collected blood culture contaminats and skin isolates produced slime. This was statistically significant, and suggested that slime mediated adherence may be a critical factor in the pathogenesis of coagulase negative staphylococci infections of medical devices.

Toshida and Ichiman (1981) studied the relationship of a capsular-type, or slime producing S. epidermidis to virulence in the mouse. They discovered that virulent strains of S. epidermidis were slime producers. So perhaps encapsulation of the organism, by slime, was a requisite of virulence for coagulase negative staphylococci, although this has yet to be shown in humans.

Marrie and Costerton (1983) studied the mode of growth of the bacteria adherent to the surfaces of various components of cardiac pacemakers infected with coagulase negative staphylococci. They found that these infecting coagulase negative strains apeared to produce more exopolysaccharide material than did the strains infecting various intravascular catheters. They attribute the differences in slime production to the degree in which fibrin and other host materials became incorporated into the developing slime.

Some researchers have looked at the ability of the coagulase negative staphylococci to adhere to different types of smooth surfaces and the effect of this adherence and growth.

Sheth et al (1983a,b) looked at bacterial adherence on two types of catheter material: teflon and polyvinylchloride (PVC). In the first study

they used an in vitro system where the catheters were briefly immersed in a bacterial suspension and then incubated overnight. They found adherence to be greater on PVC catheters than on the teflon ones. They also found that the coagulase-negative staphylococci were more adept at adhering to PVC than E. coli was. In their second study they examined intravenous (IV) catheters, over an 8 month period, for bacterial growth. They found that 6.9% of the teflon catheters were positive whereas 24.6% of the PVC catheters were positive. Also, colonization of coagulase negative staphylococci on PVC was greater than on teflon. Coagulase negative staphylococci appeared to have a greater affinity for PVC than teflon catheters.

Peters et al (1982) looked at the adherence and growth of coagulase negative staphylococci on the surfaces of IV catheters. They found that there was a progressive adherence of the organisms to catheter surfaces. These organisms were able to grow and proliferate on the inner and outer surfaces in the absence of any other externally supplied nutrients. This fact suggested that the coagulase negative staphylococci were able to use some catheter components as nutritional sources. Using scanning electron microscopy, they verified this by observing surface erosion of the catheter surrounding the growing bacterial colonies.

Hogt et al (1983) looked at the adhesion of S. epidermidis to polytetrafluorethylene-co-hexafluorpropylene (FEP), a hydrophobic material used in vascular grafts, IV catheters and trachea prosthesis. They found that the adhesion between the two was probably caused by hydrophobic bonding which was not affected by the age and metabolic state of the bacteria. Franson et al (1984) also looked at this adhesion and possible ways to inhibit it. They used both PVC and teflon catheters and found that

D-mannosamine inhibits adherence to PVC catheters. This suggested that some interaction between either the PVC catheter surface or the bacterial cell wall occurs with D-mannosamine. Alpha D-methylmannoside and N-acetyl-o-glucosamine did not inhibit the adherence.

Although it was thought that the slime merely shielded the bacteria from the affects of antibiotics and lysozyme, it now appears that slime does have some effect upon human cellular immune response. This was studied by Gray et al (1984) by measuring slimes effect upon the lymphoproliferative response of mononuclear cells to polyclonal stimulators. They found that the lymphoproliferative response to two distinct polyclonal T-cell stimulators was strongly inhibited by slime, which eventually destroyed affected cells. Since slime produced this effect only after it has been incubated with cells for some time, its action was unlikely to be mediated by rapid cytolysis. It was believed that slime may act by activating a subpopulation of cytotoxic cells which are responsible for the lysis of affected cells. Slime seemed to suppress normal host response which may prolong the course of S. epidermidis infections and increase the risk of other opportunistic infections. Borges (1982) did a study to determine the ability of human neutrophils and monocytes to adhere to two types of shunt catheters and to phagocytose bacteria. They observed that white blood cells failed to adhere in normal numbers to the catheters and failed to ingest fully a bacterial innoculum on the catheters' surfaces. While in contact with the shunt apparatus, the neutrophils also exocytosed myeloperoxidase, a major component of the intracellular microbicidal system. These observations suggested that the shunt apparatus may diminish the effectiveness of the hosts' defences at the site of implantation.

It is also possible that the surface components of these organisms may be liberated to elicit diverse biological effects. Solymossy et al (1982) reported that peptidoglycan released from cell walls inhibited the migration of leukocytes, and that lipoteichoic acid was mitogenic in lymphocyte cultures, and induced lysosomal enzyme release of macrophages. These surface components could play a role in coagulase negative staphylococci.

Baddour et al (1984) looked at the ability of 7 S. epidermidis and 4 S. hominis strains to cause prosthetic valve endocarditis (PVE) in mice. They found that S. epidermidis was able to cause endocarditis in 100% of mice, while S. hominis was able to cause PVE in 12.5% of test cases. They also observed that S. epidermidis strains were significantly more resistant to phagocytic killing in vitro then were S. hominis strains. This data suggested that there are major differences in the virulence between species of coagulase negative staphylococci, both in vivo and in vitro.

These results, from work done on the coagulase negative staphylococci, indicated that slime, adherence, and extracellular enzymes were probably the major virulence factors. However, many more studies are needed to truly understand coagulase negative staphylococci pathogenicity.

6. Typing Techniques

Sensitive typing techniques are required to subdivide groups of bacteria in order to trace source and spread of organisms during outbreaks of infection and to serve as markers for identification of virulence. Up until now the coagulase negative staphylococci had been epidemiologically studied via biotyping, phage typing and occasionally by serotyping and antibiograms. However, these typing methods attempted to identify isolates

based upon phenotypic criteria alone, and this often resulted in misleading epidemiological information.

Phage typing is a method of typing in which a phage or phages attach to a bacterial cell, enter the cell, reproduce in it, and then lyse the cell. A set of phages is usually employed to offer maximal sensitivity for differentiating the bacterial strains to be tested. The sensitivity of this method is related to the number of phage types which can be distinguished as well as to the distribution of clinical isolates within the type categories (Aber and Mackel 1981).

When phage typing was employed to type coagulase negative staphylococci, it was found that less than 60% of isolated strains were sensitive to these phages. Dean et al (1973) studied 1517 strains, and found 855 or 56% to be typeable and 44% to be untypeable. Blouse et al (1975) studied 118 epidemiologically related S. epidermidis strains, with 18 typing phages, and found 59% of these strains typable while 41% were nontypeable. Verhoff et al (1970) were able to type 75.5% of 240 coagulase negative strains, however this still meant that 24.5% were nontypable which was insufficient for epidemiological studies.

Difficulties with phage typing as listed by Aber and Mackel (1981) include the following. Susceptibility of bacteria to lysis by phage may change with environmental conditions, phenotype of the bacteria, or with changes in genetic material including plasmids. Basically a number of factors must be controlled or standardized to have a reproducible phage-typing system. Often these factors are very difficult to control, thus putting a major limitation on this method.

Biological typing or biotyping of microorganisms is based upon properties such as morphology, growth and differentiation, replication and

environmental tolerance. These tests permit the classification of organisms at the genus and species level.

The biotyping schemes developed by Baird-Parker (1974) and Kloos and Schleifer (1975) proved to be quite adequate in speciating staphylococci. However, problems did arise when these schemes were employed in epidemiological studies of the coagulase negative staphylococci.

Doern et al (1983) were able to speciate 86% of 114 coagulase negative strains using the API STAPH-IDENT system. However the biotyping of these strains was not as efficient as the speciating. Most (93.6%) of the coagulase negative staphylococci tested yielded the same biochemical profile. Gemmel and Dawson (1982) and Sewell et al (1982) both used the biotyping scheme of Kloos and Schleifer to speciate a large number of coagulase negative staphylococci strains. However both groups were unable to biotype these strains in order to do epidemiological studies. Marsik and Brake (1982) also studied biotyping of the coagulase negative staphylococci, and found Kloos and Schleifer's biotyping scheme very workable for speciation of these organisms. However they found that exact identification of these species was not always possible. Males et al (1975) studied the biotypes of clinical coagulase negative staphylococci specimens using Baird-Parker's scheme. They found that 63.9% of 228 cultures belonged to a single biotype. This data showed that biotyping has a major limitation in epidemiologic studies.

Other difficulties with biotyping as described by Aber and Mackel (1981), include the lack of reproducibility due to the lack of standardization with methods, biological variation, and media or reagent composition variation. Also the biotype is not a stable genetic property, it could be influenced by environmental conditions, and by plasmids. This

biotype instability due to plasmid exchange had been proven by Reeve and Braithwaite (1973) using klebsiella species. Reproducibility of results with biochemical tests on bacterial cultures was also looked at by Smith (1983). He reported on previous work which found discrepancies among replicate tests in the same laboratory to be 4%, and in different laboratories to be 15%. He also found large discrepancies when the same technologist read duplicate tests.

Serological typing or serotying is another method which types organisms according to phenotypic characteristics. Serotyping, according to Aber and Mackel (1981), employs many different methods. These include macro and microagglutination, the Quelling reaction, complement-fixation, counterimmunoelectrophoresis and radioimmunoassay. These techniques are basically all applications of the antigen-antibody reaction.

Serotyping has not been used in epidemiological studies of coagulase negative staphylococci, mainly because of the limitations. The conditions under which the interaction between antigen and antibody takes place are almost always unique and thus a major problem to standardize.

Antibiograms refer to the typing of microorganisms according to their antimicrobial susceptibility pattern. There are two methods available here, the disc diffusion, and antimicrobial dilution method (Aber and Mackel, 1981). These methods are standardized, reproduceable, readily available and relatively inexpensive. However they are limited by changes in antibiotic susceptibility related to environmental factors or plasmids. Another limitation would be their reduced sensitivity due to the discontinuous measuring inherent in the dilution method.

Little work has been done using antibiograms in epidemiologic studies of the coagulase negative staphylococci. Researchers such as Marsik et al

(1982) and Males et al (1975) have looked at coagulase negative staphylococci antibiograms. They concluded that these organisms are resistant to a wider spectrum of antibiotics than S. aureus, and that S. epidermidis was the most resistant species of the staphylococci. Christensen et al (1983) used the disc diffusion, antibiogram typing method to characterize clinically significant strains of coagulase negative staphylococci. Christensen found that antibiograms had very good discriminatory power, but as a typing system there were very few phenotypes. On this basis the probability of isolating any two phenotypes by chance alone would be $p>0.05$. A good typing system should have a $p<0.05$ (Aber and Mackel, 1981).

As mentioned previously sensitive typing techniques are required to biotype organisms for epidemiological studies. The methods mentioned here have proved inadequate to do epidemiological studies on the coagulase negative staphylococci. A better or more sensitive method is required to do these studies. Perhaps plasmid profiling will prove to be a suitable method.

Plasmids are cyclic, extrachromosomal double-stranded DNA molecules distinct from the cellular chromosome. They are autonomously replicating and carry genes that are not essential for host cell growth. A plasmid may provide useful genetic information to the cell in which it is located, but plasmids are usually dispensable. Plasmids can be transferred between bacterial cells by conjugation, transduction or transformation. Plasmids are similar to viruses and it has been suggested that there is an evolutionary link between the two (Norvick et al 1976). A good overview of plasmids was given by Falkow and Portnoy (1983).

By studying the plasmid profile of a given bacterial strain, one is able to fingerprint this strain. This analysis of bacteria involves taking a crude lysate of the organism and exposing it to agarose gel electrophoresis. This allows the bands of plasmid DNA to become separated according to their molecular weights. One is able to look at the resulting profiles and differentiate the organisms which are being studied.

Plasmid-pattern analysis has been successfully used as an epidemiological tool for the investigation of outbreaks of infection caused by a variety of gram negative and gram positive bacteria.

Gram negative bacteria were the first organisms to be analyzed by this method. Tompkins et al (1980) were able to prove that multiple resistance in Serratia and other enteric species were caused by the dissemination of a single R-factor plasmid. This 45-megadalton plasmid coded for ampicillin, carbenicillin, cephalothin, streptomycin, sulfonamide and aminoglycoside resistance. Markowitz et al (1983) using plasmid profiling, demonstrated that two outbreaks of Enterobacter cloacae infections in burn patients were the result of two different strains of the same organism. These infections occurred in the same burn unit, six years apart. This is but a small sample of the many cases in which plasmid profiling has been used to successfully study gram-negative infection outbreaks.

Plasmid analysis has also been used in epidemiologic studies of S. aureus infections. McGowan et al (1983) used plasmid analysis to study the spread of infections caused by strains of S. aureus resistant to gentamicin between infants in a high-risk nursery, and 20 adults in the burn unit. Lyon et al (1984) studied the molecular epidemiology of multiresistant S. aureus in geographically-separated Australian hospitals. They found that plasmid profiles from these isolates exhibited a common pattern of large

plasmids associated with resistance to gentamicin, kanamycin, tobramycin and chloramphenicol. This data showed dissemination of a multiresistant, plasmid-bearing strain of S. aureus among Australian hospitals. Lyon et al (1983) looked at nosocomial infections caused by S. aureus strains resistant to methicillin and multiple antibiotics, in Melbourne. Plasmid analysis of clinical isolates demonstrated the presence of three classes of plasmid DNA in most strains.

The coagulase negative staphylococci have also been studied using plasmid analysis with good results. Parisi and Hecht (1980) used plasmid profiling, in conjunction with phage typing, biotyping and antibiograms, in epidemiologic studies of coagulase negative staphylococci infections. In this study Parisi and Hecht looked at clinical isolates of coagulase negative staphylococci and typed each organism using these four typing mechanisms. They found that some strains had the same phage type, biotype, and antibiogram, but had distinctly different plasmid profiles. So strains which appeared identical by conventional typing mechanisms, were proved, by plasmid profiling to be nonidentical. In other cases they found strains which had almost identical plasmid profiles, the same biotype and the same phage type, but had vastly different antibiograms. Plasmid profiling enabled these investigators to show that two strains which appeared nonidentical, due to vastly different antibiograms, were in fact almost identical. The only difference was a single plasmid which altered the antibiogram results.

Archer et al (1982) looked at plasmid profiles of S. epidermidis isolates from patients with prosthetic valve endocarditis (PVE). They studied 45 plasmid containing isolates of S. epidermidis from cases of sporadic PVE from three institutions in the USA. They found that none of

these 45 isolates had similar plasmid profiles. This group also looked at 9 isolates from a cluster of PVE cases in Canada. In these 9 isolates they found two groups of three isolates which had identical plasmid patterns. This along with other clinical data suggested that these organisms were epidemiologically related. These results led Archer to conclude that in a cluster or outbreak of PVE the same strain was responsible for many of the cases. However in 45 sporadic cases of PVE, different strains were responsible for each case.

Maki et al (1982) studied methicillin resistant S. epidermidis surgical wound infections. Using plasmid profiling they were able to link these infections to a single chronic carrier The actual study consisted of a cluster of 3 sternotomy infections, following coronary artery bypass graft operations, which were caused by methicillin resistant S. epidermidis. Plasmid analysis confirmed that a single unique strain of S. epidermidis was responsible for all three cases. Archer was then able to isolate this same strain from an intern who was present during all three bypass operations. Positive identity was accomplished via plasmid analysis.

Wilson et al (1978) used plasmid profiling to study the plasmid ecology of coagulase negative staphylococci and to correlate the presence or absence of plasmids with tetracycline, chloramphenicol, neomycin, penicillin, and cadmium resistances. This study enabled them to see which strains contained zero plasmids, making these strains potential recipients in transduction, transformation, and conjugation experiments. They were also able to correlate plasmids to antibiotic resistance genes. For example they found that the tetracycline resistance gene was usually found on a 3-megadalton plasmid in the coagulase negative staphylococci.

Archer et al (1984) investigated plasmid pattern analysis for the differentiation of infecting from non-infecting S. epidermidis. Archer reasoned that repeated isolation of an organism, from the same site in a patient would suggest a probable infection. Whereas culture bacteremia due to normal skin flora would not yield the same organism upon repeated isolation, indicating no infection. Archer's results showed that out of 36 patients with documented S. epidermidis infections, 32 were found to have plasmid pattern identity among sequential S. epidermidis isolates. Archer also looked at 15 patients without documented Staphylococcus infections. He looked at two consecutive S. epidermidis contaminated blood cultures from each patient, and the plasmid profile of the contaminant. Archer found that none of these isolates had identical plasmid profiles. Archer had used plasmid profiling to differentiate infecting from non-infecting S. epidermidis successfully.

B. PLASMID CURING BY ETHIDIUM BROMIDE

Plasmid curing is the process by which a plasmid is permanently eliminated from a microorganism. This process was first documented by Hirota (1960) when he studied the effect of acridine dyes on mating type factors in E. coli. It was determined that acridine orange could eliminate F factor from an F+ culture, giving an F- culture. This F- was found to be genetically stable, showing irreversible loss of the plasmid.

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) a trypanocidal phenanthridinium has also been found to be quite effective in the curing of plasmids from microorganisms (Tomchik and Mandel 1964). Lepecq and Paoletti (1967) have described the mechanism of ethidium

bromide's effect upon nucleic acids. Ethidium bromide became bound to DNA and RNA, and inhibited DNA-polymerase and RNA-polymerase. It had no effect on protein synthesis. Ethidium bromide was intercalated between base-pairs of DNA to form a highly fluorescent complex.

Bouanchaud et al (1969) studied the elimination of antibiotic resistance in staphylococci by ethidium bromide. In this study they used S. aureus strains and compared their results with previous studies using acridine dyes as the curing agent. Hashimoto et al (1964) found penicillin and erythromycin resistance to be eliminated by acridine dyes at low frequencies: 0.1 to 3.5%. They also found penicillin resistance alone to be eliminated at a frequency of 2-8%. Bouanchaud et al (1969) looked at penicillin resistance elimination by ethidium bromide and found the frequency to be 8-100%. They also observed that much less ethidium bromide, than acridine dyes, was required to eliminate these factors. Bouanchaud et al (1969) also observed that in four-strains of S. aureus penicillin resistance was never eliminated. This, he concluded, was because the resistance genes were on the chromosomal DNA, not the plasmid DNA. A final observation by Bouanchaud et al (1969) was that different strains of bacteria will exhibit different sensitivities to ethidium bromide. Differences in DNA polymerase and RNA polymerase sensitivities were probably responsible for this.

Other investigators have also used plasmid elimination by ethidium bromide to aid their studies. Bouanchaud et al (1969) looked at plasmid elimination in Enterobacteria. They found plasmid elimination in gram negatives to be less efficient than it is in gram positive bacteria. Vogel et al (1969) studied infections due to a gentamicin resistant S. aureus strain in a nursery. Using plasmid elimination by ethidium bromide

treatment they were able to correlate gentamicin resistance genes to a 11 megadalton plasmid, i.e. when this plasmid was eliminated from the bacteria, the organism became gentamicin sensitive.

So it appears that ethidium bromide is a powerful drug in eliminating plasmids at a high frequency, and would appear to be a useful tool for further study in elimination of plasmid genes from microorganisms.

C. TRANSFORMATION OF PLASMID DNA

DNA can be transferred within and between genera by three different mechanisms, transformation, transduction, and conjugation.

Transformation is defined as the ability of bacteria to acquire a heritable altered phenotype by the acquisition of DNA from an external medium (Deich and Smith, 1980). Chromosomal fragments or plasmid DNA may be transformed.

Transformation was first discovered by Griffith in 1928 using Streptococcus pneumoniae. Griffith was able to transform rough avirulent cells into smooth virulent cells (Stanier et al 1976).

Avery et al (1944) succeeded in purifying pneumococcal transforming principle and identified it as DNA. Until this time it was believed that a protein moiety was responsible for the specificity of a gene This was the first evidence that DNA was the carrier of genetic information. Since 1944 transformation has been effected in other genera of bacteria. Haemophilus, Neisseria, and Bacillus all proved to be naturally transformable, while other genera had to be made competent before transformation could succeed. Competence is defined as the ability of a bacterial strain to take up DNA and undergo transformation. The competent state requires genetic

information and special physiological conditions in order to obtain a high frequency of transformation. E. coli can only be transformed in the presence of specific cations and a heat-shock regime for the uptake of exogenous DNA.

The transformation process had been extensively studied in the gram-positive pneumococci. There are three recognized stages in this process. 1) Double-stranded DNA fragments bind to sites on the recipient cell, that are present only during competence, 2) the cell was committed to uptake of the bound DNA molecule, 3) duplex DNA was transported into the cell. The limiting factor in the yield of transformants was usually the competence of the recipient cell population to take up transforming DNA (Stanier et al 1976).

The staphylococci, like E. coli must be made competent before transformation can occur. Rudin et al (1974) studied the variables affecting transformation of both plasmid and chromosomal markers in S. aureus. They found the optimal pH and temperature for transformation are 6.75-7.0 and 30^{o}C, respectively. Calcium ions were required for transformation. Maximal numbers of transformants were obtained after 20 minutes of contact between cells and DNA. Lindberg et al (1972) found that recipient cells showed competence throughout the exponential growth phase with a maximum at early times.

Several researchers have successfully performed transformation on the staphylococci, although most of this work was done on S. aureus. Lindberg and Novick (1973) successfully transformed a penicillinase plasmid in S. aureus cells. Forbes and Schaberg (1983) described the transfer of resistance plasmids between S. epidermidis and S. aureus. Jaffe et al (1980) described the transfer of gentamicin resistance plasmids between S. aureus and S. epidermidis.

So under the right conditions, transformation may be used to study the transfer of genes (DNA) between staphylococci species.

D. ANTISERUM AGAR

If an antigen and the corresponding precipitating or flocculating antibody diffuse towards each other in an indifferent gel, such as agar, there will appear, under certain circumstances a streak or bandlike precipitation in the gel between the two diffusing components. The reaction is of the same nature as that between antigen and antibody. A similar reaction is obtained if antigens are allowed to diffuse in gels with a suitable constant proportion of antibodies or vice versa (Ouchterlony, 1948). It is this basic principle which formed the basis of the antiserum agar technique.

Kirkbride and Cohen (1934) were one of the first groups to describe this technique. They worked on Meningococcus strains and described the halo phenomena around bacteria colonies growing on agar plates containing the specific immune serum. Since this time many investigators have used this technique. Most of the work has been done in the last two decades. Bradshaw et al (1971), studied bacterial antigens cross-reactive with the capsular polysaccharide of Haemophilus influenza type b. They used agar plates containing anti Haemophilus influenza type b serum, and grew various bacterial colonies isolated from the pharynx and rectum of humans and rabbits. A halo of precipitation was observed surrounding some colonies, and this halo was shown to be due to polysaccharides cross-reactive with the type b polysaccharide of H. influenza. Schneerson et al (1972) did very similar work using the antiserum agar technique. They showed that E.

coli contained an antigen that was cross-reactive with the capsular polysaccharide of H. influenza type b.

Michaels and Stonebraker (1975) used the antiserum agar technique for detection of H. influenza type b in the pharynx of infants and young men. They used antiserum agar medium to determine its stability and accuracy in this study. They found that the medium could be stored at $4^{o}C$ for periods of up to 12 weeks with no loss of effectiveness. Also it was shown that all halo-producing organisms detected after 24-40 hours of incubation were H. influenza type b. This group also observed that this technique was dependent on large amounts of potent and highly specific antiserum. West et al (1985) used the antiserum agar method for identificaiton of Smith type exopolysaccharides in clinical isolates of S. aureus. They obtained 140 S. aureus blood isolates and grew them on the agar containing the antiserum to the S. aureus Smith diffuse strain. They found that ninety (64.3%) of the 140 isolates produced precipitin halos on the antiserum agar. This technique enabled them to prove that the presence of clinical S. aureus strains producing exopolysaccharides antigenically identical to the Smith diffuse strain exopolysaccharide was a common phenomenon.

The antiserum agar technique appears to be an accurate and stable method, which is dependent upon large amounts of potent and highly specific antiserum being present in the agar.

MATERIALS AND METHODS

A. Bacterial Strains

All but one strain of coagulase negative staphylococci used in this study were obtained from clinical isolates, stocked in the department of Microbiology, St. Boniface Hospital at -70°C. The one exception was a strain obtained from Dr. Archer (U. of Maryland, Washington, D.C.). This was designated Sanders strain, and was known to contain five plasmids. Eschericha coli strains used were obtained from stocks kept by L. Slaney (Department of Medical Microbiology, U. of Manitoba).

B. Culture Media and Growth Conditions

1. Solid Media

Coagulase-negative staphylococci species were maintained on Sheep Blood Agar (Gibco Laboratories, Madison, Wisconsin). Cultures were incubated aerobically at 37°C for approximately 18 hours. E. coli strains were maintained on Sheep Blood Agar under growth conditions described above.

When appropriate, selective media was prepared. Mueller-Hinton Agar (Gibco) was supplemented with a combination of Sulfamethoxazole (Hoffman-LaRoche Limited, Montreal, Canada) and Trimethoprim Lactate (Burroughs Wellcome Limited, LaSalle, Quebec) in a ratio of 20:1 respectively. These drugs were added to a final concentration of 128 mg per litre of medium. These two drugs combined are abbreviated SXT.

2. Liquid Media

When grown in liquid medium, coagulase-negative staphylococci were grown in Tryptic Soy Broth (TSB) (Gibco). Cultures were incubated aerobically at 37°C, with or without shaking. E. coli strains were grown in Brain Heart Infusion Broth (Gibco), under the growth conditions described above. When appropriate antibiotics were added to the liquid medium at the following concentrations. Streptomycin (Allan and Hanburys, Glaxo Canada Limited, Toronto) 500 ug per ml and tetracycline (Sigma Chemical Company, St. Louis, Missouri) 4 ug per ml of medium.

C. Taxonomic Identification of Bacterial Strains

Coagulase-negative staphylococci used were identified using the following criteria, a) colonial morphology b) gram stain c) catalase reaction d) coagulase production and e) biochemical profiles using api STAPH-IDENT strips.

D. Stock Preparations

Stock preparations of all bacterial strains used were prepared by inoculating a large loopfull of bacteria into 2 mls of skim milk. These were stored at -20°C or at -70°C.

E. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done by the disc susceptibility method or by the minimum inhibitory concentration method. Both of these

methods were described by Ronald et al (1969). All antibiotic susceptibility testing was done by the clinical microbiology lab in the Health Sciences Centre, Winnipeg, Manitoba.

F. Plasmid Profiling

Various rapid plasmid isolation methods were employed in this study. However, it was found that a method by Cooksey (1981), Centers for Disease Control, (CDC), Atlanta, Georgia, was adequate following a few alterations.

In this method a large loopfull of coagulase-negative staphylococci were removed from a blood agar plate and suspended in 200 ul of TRIS-EDTA saline buffer (TES), (Appendix A). Then 50 ul of lysosotaphin (Sigma) (1 mg/ml TES) was added, and after gently mixing, the tube was incubated at 37^{o}C for 30 minutes in a water bath. After incubation 400 ul of TES containing 8% sodium dodecyl sulfate (Fisher Scientific Company, New Jersey) was added to the tube. The tube was now incubated for twenty minutes in a 68^{o}C water bath, with gentle inversion every five minutes. The sample was then placed on ice for fifteen minutes, or until frozen. After freezing the tube was given five spins of two minutes each at 12,800 x g in a microfuge. Each spin was followed by five minutes in an ice bath. After these five spins the top half of each tube contained a clear supernatant. This supernatant was transferred to a fresh 1.5 ml microfuge tube and one tenth volume of 3M anhydrous sodium acetate (Fisher) was added along with two volumes of cold absolute ethanol. This was done to precipitate the DNA out. The tube was now placed at -70^{o}C for 30 minutes. The DNA was then pelleted by centrifugation for seven minutes in the microfuge. The supernatant was poured off, and the pellet was air dried.

After the pellet dried, the alkaline procedure was applied to further enhance the purity of the plasmid DNA (Vinograd et al, 1966). The pellet was gently resuspended in 200 ul of TES, and then 50 ul of 0.3M sodium hydroxide (Fisher) was added. The tube was gently mixed and left at room temperature for 10 minutes. After 10 minutes had passed 20 ul of 2M TRIS (Sigma), pH 5.1, was added, followed by 50 ul of 0.25 M EDTA (Sigma) pH 8.1, and 50 ul of 3M sodium acetate pH 9.0. The plasmid DNA was then precipitated by adding two volumes of absolute ethanol and incubating for 30 minutes at $-70^{o}C$. The DNA was pelleted by spinning for five minutes in the microfuge. The supernatant was decanted and the pellet was air dried. After drying the pellet was resuspended gently in 100 ul of TES for future agarose gel electrophoresis.

G. Agarose Gel Electrophoresis

1. Vertical Gel Electrophoresis

Vertical gel electrophoresis of plasmid DNA was carried out on a Bethesda Research Laboratories Inc. (Rockville, Maryland), Model V-16-2 electrophoresis system. Gels were run in TRIS-BORATE BUFFER (Appendix A) at 35 mAMP and 100 volts for 3 hours or until the tracking dye had migrated to within 2 cm of the bottom of the gel. A 50 ul aliquot of the sample was taken and 15 ul of tracking dye (Appendix B) was added to it. Then 60 ul of this sample was placed in a single lane of the gel. All gels run had a final concentration of 0.7% agarose (Seakem Agarose, Mandel Scientific Col, Rockwood, Ontario) in TRIS-BORATE Buffer. After completion of the gel run, the DNA was visualized within the gel matrix by staining in an ethidium bromide (Sigma), double distilled (dd) water solution. The staining

solution had a final ethidium bromide concentration of 3 ug per ml. After staining the DNA bands were visualized by illuminating the stained gel with a Chromoto-Vue Transiluminator (model 0-61, Ultra-Violet Products Inc., San Gabriel, California). The illuminated gel was then photographed with a Polaroid MP4 camera using Polaroid type 57 high speed film.

2. Horizontal Gel Electrophoresis

The procedure used for horizontal gel runs was identical to the procedure used for vertical runs with the following exceptions. The apparatus used for horizontal gel runs was acquired from the Aquebugue Machine and Repair Shop, Aquebugue, New York. Also this system employed larger lanes, thus up to 200 ul of sample plus tracking dye could be applied per lane.

H. Plasmid Molecular Weight Determination

The molecular weights of the various plasmids isolated from the coagulase-negative staphylococci strains were determined by comparing their migration in a gel matrix to the migration of plasmids with known molecular weights. After determining how far the test plasmids had migrated, the molecular weights were determined by plotting the logarithm of the relative distance migrated of standard plasmid DNA preparations through the gel, versus logarithm of the plasmid molecular weight.

The 5 plasmids used as molecular weight standards were isolated from Cesium chloride (CsCl) gradients using the method of Myers et al (1976). These plasmids ranging in size from 1.8-6.2 x 10^6 daltons were isolated from 5 separate E. coli strains. These strains were grown overnight in 400

ml of Brain Heart Infusion broth with continuous shaking. The bacterial cells were then harvested by centrifugation in an IEC model B-20A centrifuge at 30,000 x g for 20 minutes. The pellet was washed in 20 ml of TES buffer, and repelleted by centrifugation at 30,000 x g for 20 minutes. The pellet was then resuspended in 2 ml of cold TRIS-sucrose buffer (Appendix A). A 0.4 ml aliquot of freshly prepared lysozyme (Sigma) in 0.25M Tris pH 8.0 (5 mg/ml) was added, and this was incubated at room temperature for 5 minutes. A 0.8 ml aliquot of 0.5M EDTA pH 8.0 was added and this was incubated for 30 minutes at room temperature. Then a 4.4 ml aliquot of Triton-lytic Mix (Appendix A) was added, and after gentle inversion, incubation was continued for 30 minutes at room temperature. After incubation the mixture was centrifuged at 120,000 x g at $4^{o}C$ for 30 minutes. The clear supernatant, enriched in plasmid DNA, was saved and transfered to a polyallomer tube (No. 326814, Beckman Instruments Inc., Fullerton, California). To each polyallomer tube, 1 gm CsCl (Beckman) per ml of supernatant was added. The CsCl was completely dissolved by gentle agitation. The samples were then moved to a dark room where 0.2 ml of ethidium bromide solution (10 mg per ml of TES) was added. The tubes were topped off with parrafin oil (Fisher), capped, balanced, and centrifuged for 40 hours at $18^{o}C$ at 150,000 x g in a Beckman Model L5-65 ultracentrifuge with a Beckman 50Ti fixed angle rotor. Following centrifugation the tubes were carefully removed from the rotor in a dark room. The plasmid DNA bands were visualized under long wave ultra-violet light (Black-Ray Lamp, Model UVL-21, Ultra-Violet Products Inc., San Gabriel, California) and collected by dripping the gradient from the tube using a Beckman Fraction Recovery System. The ethidium bromide was extracted from the plasmid DNA with 3 changes of CsCl-saturated isopropyl

alcohol. The plasmid DNA was then dialysed at 4°C against 10mM Tris-1mM EDTA for 24 hours. After dialysis, one tenth volume 3M sodium acetate was added to the plasmid DNA. The DNA was then precipitated with two volumes absolute ethanol at -70°C for 30 minutes, and pelleted by centrifugation at 12,000 x g for 20 minutes at -10°C. The pellet was air dried and resuspended in TES buffer.

I. Slime Detection

The detection of slime production by the coagulase-negative staphylococci was determined by the method developed by Christensen et al (1982). The organism to be tested was grown in 7 ml of Tryptic Soy Broth, for 18 to 24 hours at 37°C, in a 13 ml glass conical tube (Pyrex Glass Company, USA). Tryptic Soy Broth contains glucose (0.25% wt/vol) and casein digests which are required for expression of adherent growth. The organism was grown without agitation. Following incubation the culture media was decanted, and the inner surface of the tube was examined for the presence of slime. Ring formation at the liquid-air interface was not considered to be indicative of slime production by this particular coagulase-negative staphylococci strain. If a film of slime was not detectable, 1 ml of an alcian blue dd H_2O solution (100 ug/ml) was washed over the surface of the tube. If the inner surface of the tube retained the stain there was slime production.

J. Plasmid Curing

1. Minimum Inhibitory Concentration

Prior to attempting to cure a plasmid from a bacterial strain the minimum inhibitory concentration (MIC) of the curing agent must be determined (Bouanchaud et al, 1969). The curing agent used was ethidium bromide. The MIC was considered the lowest concentration of ethidium bromide which did not allow macroscopic growth of the coagulase-negative staphylococci strain. The procedure employed was adapted from work by Bouanchaud et al (1969). Five ml of Tryptic Soy Broth was put into a 50ml pyrex tube, and the appropriate amount of ethidium bromide was added. Ethidium bromide concentrations ranged from 6 uM to 400 uM. A 0.1ml aliquot of a 10^{-5} dilution of a fresh overnight culture was added to broth plus ethidium bromide tube, and this was incubated at 37°C, with agitation overnight, and subsequently examined for macroscopic growth. The first concentration below the MIC was chosen for subsequent curing experiments.

2. Curing Methodology

Curing experiments were carried out using a modified procedure of Bounchaud et al (1969). After determining the appropriate ethidium bromide concentration the following procedure was done. A loopful (0.01 ml) of the broth culture was streaked on a 5% sheep blood agar plate and incubated at 37°C overnight. Single colonies were picked off the plate, restreaked onto blood agar plates, and incubated at 37°C overnight. These plates were removed from the incubator and lysates for plasmid profiling were prepared by the method previously described. A lysate was also prepared of the strain being tested, before curing was attempted. Now agarose gel

electrophoresis was carried out with all of the lysates. After photographing the gel, the cured lysates were compared to the original strain to see if the plasmid had been cured from any of the ethidium bromide treated bacteria.

K. Plasmid Isolation from an Agarose Gel

The technique of isolating a plasmid from an agarose gel was published by Maniatis (1982), and adapted to the coagulase negative staphylococci organisms. A lysate of the desired organism was prepared, the sample was loaded onto the horizontal gel, and eletrophoresed at 100 volts. Once the tracking dye (Appendix B) had traveled down one half of the gel the power was disconnected, and the gel was removed from the apparatus and placed in ethidium bromide staining solution for 10 minutes. After staining, the gel was carefully returned to the electrophoresis apparatus. Under the electrophoresis system the Chromato-Vue Transilluminator (Model 0-61) was placed to illuminate the stained gel. The plasmid band could be seen in the stained gel. Using a sharp scalpel or razor blade, a trough was cut directly in front of the leading edge of the plasmid DNA band, and about 2 mm wider than the band on each side. Incisions were also made perpendicular to the trough, which extended 2 mm above the DNA band. Using forceps this piece of gel was lifted and a piece of dialysis tubing was inserted under the gel, through the trough and over a section of the uncut gel. The trough was filled with electrophoresis buffer, and electrophoresis was resumed. Every 2 or 3 minutes the fluid was recovered from the trough and fresh buffer was put into the trough. This was continued until all the DNA in the band had been removed from the gel. The

current was then reversed for 2 minutes to remove any DNA from the dialysis tubing, and this DNA was recovered from the trough. The DNA was then purified by precipitating with one tenth volume sodium acetate, and two volumes absolute ethanol. This was incubated at $-70^{o}C$ for 30 minutes, followed by precipitation at 12,000 x g for 20 minutes at $-10^{o}C$. The pellet was air dried and resuspended in TES buffer.

L. Transformation

A coagulase negative staphylococci strain was found, via plasmid profiling, to contain no plasmids. This strain was chosen to be the recipient cell for transformation. The plasmid DNA to be transformed was isolated as previously described. This plasmid was known to contain penicillin and SXT resistance genes.

Methodology used was adapted from a paper by Lindberg and Novick (1973). Competent cells, approximately 10^{9} colony forming units per ml, in 0.9 ml of 0.1M TRIS-HCl (Sigma) pH 7.0 containing 0.1M calcium chloride (Sigma), were mixed with 0.1 ml of 10 ug DNA per ml TES solution. After twenty minutes of contact between the bacterial cells and DNA at $27^{o}C$, the mixture was centrifuged at 11,000 x g for 20 minutes. The supernatant was discarded, and the cells were resuspended in 1 ml of Tryptic Soy Broth. The mixture was then left for 2 hours at $37^{o}C$ to allow for phenotypic expression of the resistance markers. After 2 hours 0.1 ml aliquots were spread on Mueller-Hinton agar plates containing 12.8 mg SXT per 100 ml. The plates were then incubated for 18 hours at $37^{o}C$. Colonies which grew were checked for transformation via plasmid profiling and agarose gel electrophoresis. Controls consisted of competent cells, and the original

coagulase-negative staphylococci strain containing the transformed plasmid.

M. Slime Isolation

Slime isolation was carried out using the modified procedure of Murthy et al (1983). The coagulase-negative staphylococci strain used was a heavy slime producer. This strain was grown overnight in 2 liters of Tryptic Soy Broth at 37°C, on a rotary shaker. Cells were pelleted by centrifugation at 10,000 x g for 30 minutes. The pellet was resuspended in 50 ml of 10% trichloroacetic acid (Sigma) and heated at 85-95°C for 30 minutes with magnetic stirring in a 500 ml erlenmeyer flask. Cellular debris were pelleted by centrifugation at 25,000 x g for 40 minutes. The pellet was discarded and the yellowish-brown supernatant was dialysed using three quarter inch seamless cellulose tubing (Fisher) for 48 hours at 4°C against several changes of dd water in a 1 liter erlenmeyer flask. The dialysed material was then evaporated to dryness in vacuo using a lyophilizer (Virtis Research Equipment, Gardiner, New York). The residue was redissolved in 2 ml dd water, and 8 ml of absolute ethanol, room temperature, was slowly added. The resulting precipitate was then collected by centrifugation at 25,000 x g for 25 minutes at 4°C. The supernatant was discarded and the pellet was carefully dissolved in 1 ml 0.5 M sodium chloride. To precipitate the polysaccharide 4 ml of absolute ethanol, room temperature, was added slowly. The polysaccharide was pelleted by centrifugation at 25,000 g for twenty minutes at 4°C. The polysaccharide was then dissolved in 4 ml dd water and dialysed against several changes of dd water in a 1 liter erlenmeyer flask for 48 hours. This gave a crude preparation which could be further purified by ion exchange chromatography.

N. Ion Exchange Chromatography

Ion exchange chromatography was performed to remove contaminating proteins and teichoic acids. Dry QAE-Sephadex A-50 beads (Pharmacia Inc., Toronto, Canada) were soaked overnight in 0.01 M hydrochloric acid (Fisher), and a column (1 x 17cm, packed dimensions) was prepared. The crude polysaccharide, 100 mg, was dissolved in 3 ml 0.01 M hydrochloric acid, and any insoluble material was removed by centrifugation at 10,000 x g for 10 minutes. After equilibrating the column with 3 volumes of 0.01 M hydrochloric acid, the sample was loaded onto the column. The sample was then eluted stepwise with 3 column volumes each of 0.01 M hydrochloric acid, 0.25 M sodium chloride, and 0.5 M sodium chloride. The capsular polysaccharide was eluted from the column in the 0.5 M sodium chloride eluate. Salt was removed from this fraction by dialysis against dd water at $4^{o}C$ for 48 hours in a 1 liter erlenmeyer flask with several changes of dd water. The sample was then lyophilized, leaving a white fluffy product.

O. Thin-Layer Chromatography

Thin-layer chromatography analysis was adapted from a paper by Murthy et al (1983). Thin-layer chromatography was performed on T-6770 polyester silica gel plates (Sigma). The solvent system used was a mixture of pyridine, butyric acid, and water. This solvent contained 5 parts pyridine, 3 parts butyric acid, and 1 part water. Each of the three fractions eluted from the column was analyzed by thin-layer chromatography. The three fractions were analyzed in the form they were eluted, as well fractions two and three were analyzed after being subjected to acid

hydrolysis. Acid hydrolysis consisted of taking 5mg of sample, and 1 ml of 1M sulfuric acid (Fisher) and sealing both in vacuo into a 2 ml ampule type tube (Fisher). The tube was heated at 100°C for 3 hours, followed by neutralization to pH 7.0 with barium carbonate (Fisher). The barium carbonate precipitated out leaving a clear supernatant containing the hydrolyzed polysaccharide. This was then lyophillized to give the hydrolyzed polysaccharide in the dry form (Hellerquist and Ruden, 1972). Prior to thin-layer chromatography, 1 mg of all samples to be analyzed were mixed with 1ml dd water to give a 1mg per ml solution. When the sample was applied to the plate 5 ul or 5 ug was applied. The plates measured 10cm x 10cm and samples were applied 1cm from the bottom of the plates. The plates were left in the solvent apparatus for approximately 2 hours or until the solvent front was within 1 cm of the top of the plate.

P. Immunization Procedure

Two rabbits were immunized with the isolated slime plus Freunds complete adjuvant (Sigma) according to a schedule listed by Hurn and Chantler (1980). Initially the rabbits were immunized with 0.1 mg of slime plus 0.9 ml Freunds complete adjuvant. Booster shots containing 0.05 mg slime plus 0.95 ml Freunds complete adjuvant were given every 21 days for a period of 84 days. All injections were given intradermally. Prior to the booster shots, 40 ml of blood was obtained from the rabbits.

The blood collected from the rabbits was stored in 50ml conical centrifuge tubes (Falcon Plastics, Oxnard, California), overnight at 4°C. Sera was obtained by centrifugation at 1500 x g for 10 minutes. The sera contained the immunoglobulins. The sera was stored in a sterile 50 ml

conical tube at -20°C until further use.

Q. Preparation of Antiserum Agar Plates

1. Precipitation of Immunoglobulins

This procedure was published by Stelos (1978). Immunoglobulins were precipitated out of solution by the addition of saturated ammonium sulphate (Sigma). One volume saturated ammonium sulphate was added to every two volumes sera to begin the procedure. The ammonium sulphate was added slowly with constant stirring. This mixture was left standing for 15 minutes. The sediment was saved and washed twice in 50 ml of 40% saturated ammonium sulphate, then redissolved in 50 ml of 0.9% sodium chloride. Saturated ammonium sulphate (25 ml) was slowly added to precipitate out the immunoglobulins again. This sediment was then washed with 40 ml of 40% saturated ammonium sulphate, and resuspended in 50 ml of 0.9% sodium chloride. Immunoglobulins were again precipitated with 25 ml saturated ammonium sulphate. This was followed with two washings in 50 ml of 40% saturated ammonium sulphate. Finally the sediment was resuspended in 40% saturated ammonium sulphate and stored at 4°C indefinitely.

2. Antiserum Agar Plates

Antiserum agar plates were prepared using a procedure adapted from papers by Bradshaw et al (1971) and Michaels and Stonebraker (1975). To begin 9.25 g of brain heart infusion broth plus 5.0 g agarose (Seakem) were combined with dd water to a final volume of 250 ml. This was sterilized and 50 ml of the antiserum, equilibrated in a waterbath at 50°C, was added. Then 15 ml of this medium was poured per rodac plate (Falcon), allowed to

gel, and stored in sealed plastic bags at 4°C until required.

R. Congo Red and Alcian Blue Plates

Congo red plates, to study the capsular polysaccharide of the coagulase-negative staphylococci, were prepared as follows. To begin 0.02 g of Congo Red (Sigma) was combined with 2 liters of Tryptic Soy agarose to give a final Congo Red concentration of 100 ug per ml. This medium was poured into petri plates (Fisher), approximately 35 ml per plate, allowed to gel, and stored in sealed plastic bags at 4°C until required.

Alcian blue plates were prepared as above to a final concentration of 800 ug alcian blue (Sigma) per ml of Tryptic Soy agarose.

TABLE II. Coagulase-negative staphylococci organisms used.

Stock Number	Species Identification[1]
7722-1	S. capitis
4571	S. epidermidis
4625	S. epidermidis
6685	S. haemolyticus
6730	S. haemolyticus
4595	S. hominis
4605	S. hominis
5088	S. simulans
6007	S. simulans
4555	S. warneri
7376	S. warneri
7092	S. xylosus
128	S. epidermidis
129b	S. epidermidis
4485	S. epidermidis
6081	S. epidermidis
4882	S. epidermidis
14	Unknown
61	S. epidermidis
49	S. epidermidis
155	S. epidermidis
180	S. epidermidis
587	S. epidermidis
870	S. epidermidis
1046	S. epidermidis
6008	S. epidermidis
129h	S. epidermidis
6113	S. epidermidis

[1]Species identification determined with api STAPH-IDENT strips.

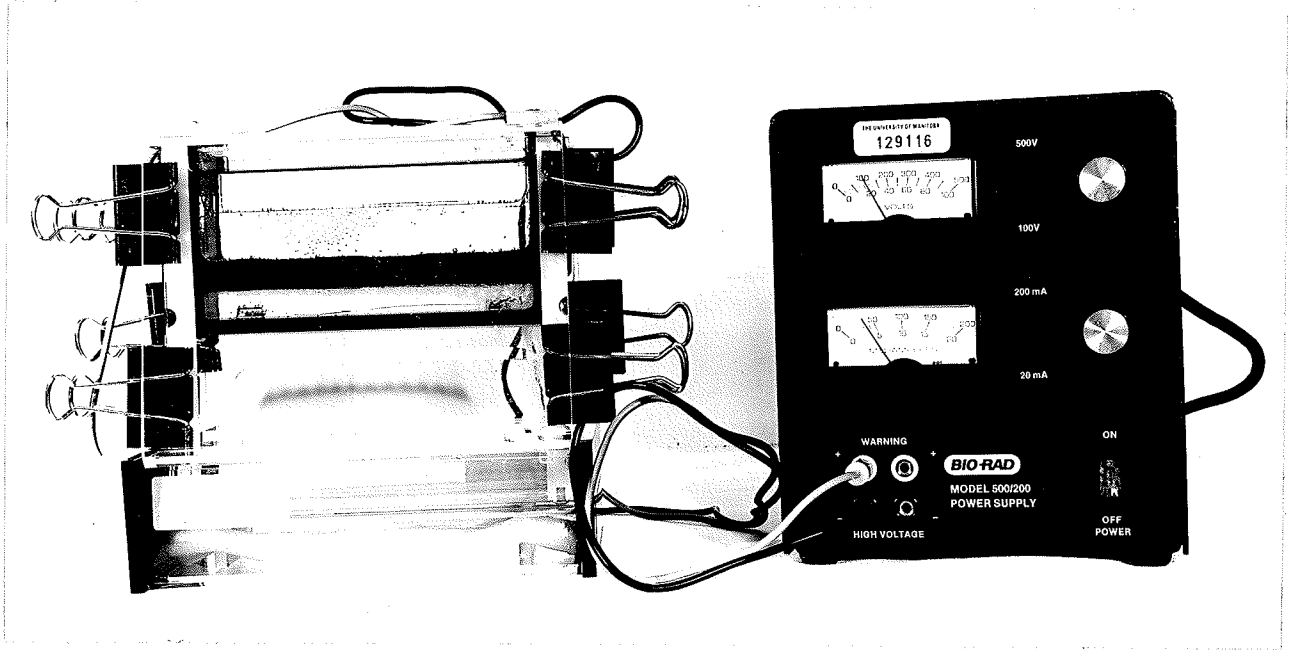

Figure 1: Agarose gel electrophoresis apparatus.

RESULTS

A. Plasmid Profiling Method

Initial results from the plasmid profiling of the coagulase-negative staphylococci were disappointing. Using many different methods, including methods reported by Cooksey (1981), Kloos et al (1980), Holmes and Quigley (1980), and Forbes and Schaberg (1983), it was found that the gels were consistently contaminted with chromosomal DNA. This is clearly illustrated in Figure 2. This contamination masked the detection of plasmid DNA bands in the lanes of the gel.

After this, it was decided to settle on one method and make changes to improve electrophoresis results. The method employed was the one published by Cooksey (1981) and the changes made are listed in Table III. Although the quality of the results was greatly improved there was still minor contamination with chromosomal DNA and plasmid artifacts, so a procedure developed by Vinograd and Lebowitz (1966) was applied. This alkaline treatment eliminated the contamination, leaving a gel in which the results could easily be interpreted. The improvement implemented by the alkaline treatment is shown in Figure 3.

This method was then applied to six coagulase-negative staphylococci strains over a period of three weeks to see if good and consistent results could be achieved. It was found that the desired results were received, Figure 4, and thus a good plasmid profiling method for the analysis of the coagulase-negative staphylococci had been developed. This method, and the reasoning behind the major steps are listed in Table IV.

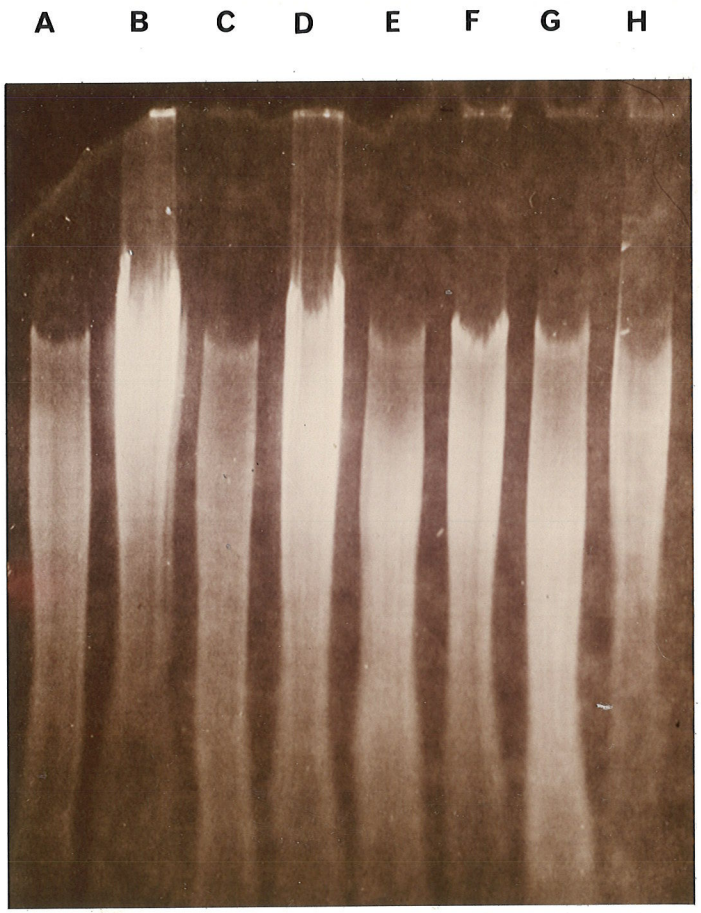

Figure 2: Agarose gel (.7%) of plasmid DNA isolated from 8 coagulse-negative staphylococci organisms demonstrating extreme chromosomal contamination. This gel was done using Cooksey's (1981) method. Lanes A-H respectively contain organisms 587, 1046, 870, 61, 6008, 155, 49, and 180.

TABLE III. CDC Method Before and After Alterations

Procedure	CDC	Altered CDC
Growth Media	Tryptic Soy Agar containing 0.3% yeast extract and 0.5% glycine	Sheep Blood Agar
Lysostaphin concentration	30 ul of a 1 mg/ml TES solution	50 ul of a 1 mg/ml solution
SDS Addition	250 ul TES containing 8% SDS	400 ul TES containing 8% SDS
65^{o} water bath	10 minutes	20 minutes
Alkaline Procedure	Not done	Yes

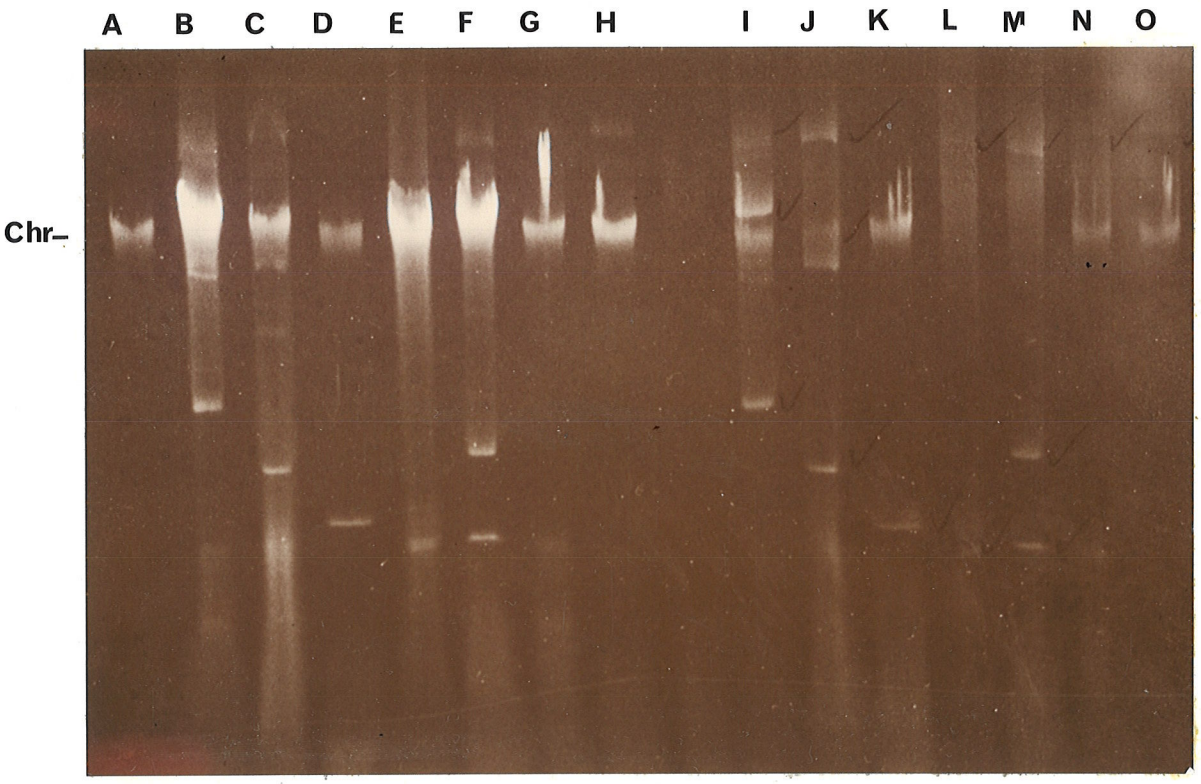

Figure 3: Agarose gel (.7%) of plasmid DNA isolated without, and with alkaline treatment. Lanes A-H are without alkaline treatment. Lanes I-O are with alkaline treatment. Lanes A-H, and I-O respectively contain organisms numbered 587, 1046, 870, 61, 6008, 155, 49, and 180. CHR is chromosomal DNA, all other bands represent plasmid DNA.

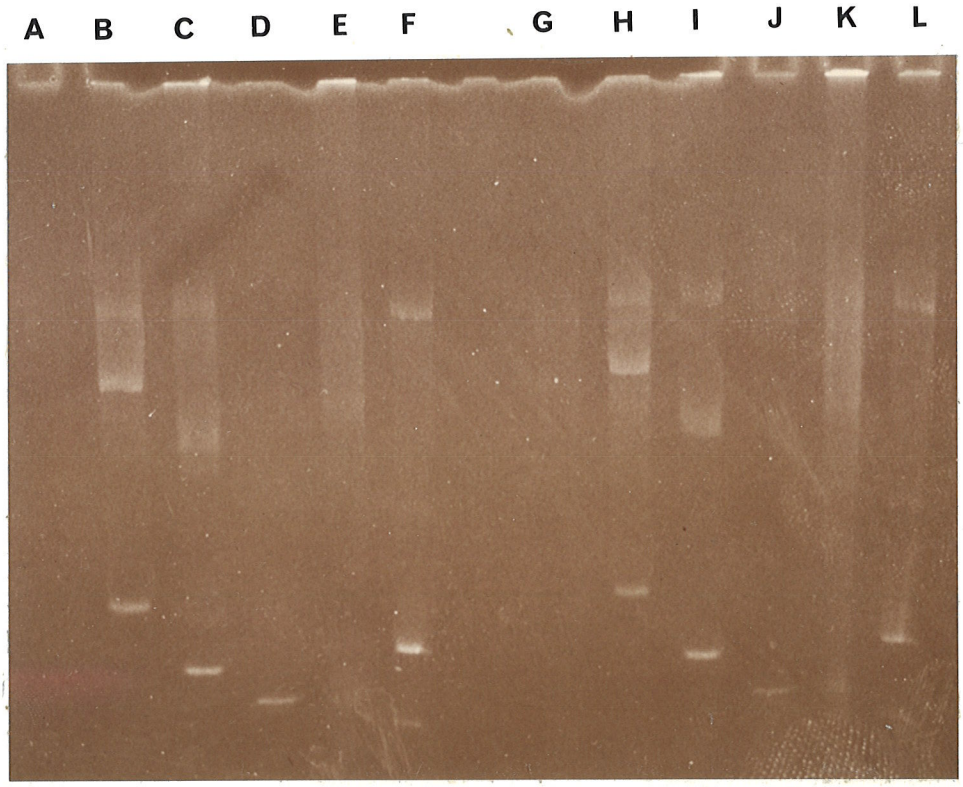

Figure 4: Agarose gel (.7%) of plasmid DNA isolated from 6 coagulase-negative staphylococci strains. Lanes A-F, and Lanes G-L respectively contain organisms numbered 587, 1046, 870, 61, 6008, and 155. These were done using the final altered Cooksey method.

TABLE IV. Final CDC Method and Reasoning Behind Method.

Methodology

1. Scrape a large loopful of cells from a Sheep Blood Agar plate and suspend in 200 ul of TES containing 20% sucrose.

2. Add 50 ul of lysostaphin (1 mg/ml TES) and mix gently, incubate at 37°C for 1/2 hour.

3. Remove from H_2O bath and add 400 ul of TES containing 8% SDS.

4. 20 minutes in 68°C H_2O bath.

5. Put in ice for 15 minutes and then spin in microcentrifuge for 2 minutes and then place in ice water for 5 minutes. Repeat this approximately 5 times or until 1/2 of the tube is a clear supernatant.

6. Collect supernatant and add 1/10 vol 3M NaAc and 2 x vol EtoH and place at -20°C overnight or -70°C for 30 minutes to achieve precipitation.

7. Next day spin lysate 5 minutes in microcentrifuge and pour off supernatant.

8. Resuspend in 200 ul of TES and apply alkaline treatment:
   - add 50 ul of 0.3 M NaOH for 7 minutes
   - add 20 ul 2 M Tris, pH = 5.1
   - add 50 ul .25 M EDTA, pH = 8.1
   - add 50 ul 3 M NaAc, pH = 9.0

   and then 2 x vol EtoH and precipitate at -70°C for 1/2 hour.

   Spin in microfuge for about 7 minutes and then pour off supernatant, and allow precipitate to dry.

   Resuspend in 100 ul of TES and 25 ul of tracking dye. The sample is now ready to be added to a 0.7% agarose gel and to undergo electrophoresis for about 3 hours at 100 volts.

   After electrophoresis the gel is stained for 10 min. in a ethidum bromide solution and then photographed under a UV light.

TABLE IV. (Cont'd)

Reasoning behind these steps:

1. Sucrose is added to prevent immediate osmotic lysis.

2. Lysostaphin is added to punch holes in the cell wall to form protoplasts.

3. Controlled lysis is achieved via the addition of SDS, this allows cell contents to leak out of protoplasts. The majority of chromosomal DNA should remain inside the protoplast because its attached to cytoplasmic membrane.

4. "Debris" is removed via $68^{o}C$ treatment.

5. Alkaline treatment degrades linear DNA (chromosomal) whereas it does not effect the closed circular plasmid DNA.

B. Application to Staphylococcus species and Plasmid Stability

In order for this method to be considered useful in the study of coagulase-negative staphylococci organisms it must be applicable to all species in this genera. Twelve coagulase-negative staphylococci strains were obtained from St. Boniface Hospital in order to show this. These organisms are listed in Table II. Only seven of the ten recognized human coagulase-negative staphylococci species were represented due to availability of these organisms. S. cohnii, S. auricularis and S. saprophyticus were not included in these tests. The results showed that all of the organisms tested were lysed by this procedure to give lysates which when applied to gel electrophoresis could be easily interpreted. This is shown in Figures 6, 7 and 8. Although lanes 4, 5, 10, and 11 revealed no plasmids, there was evidence of chromosomal DNA which indicates that lysis did occur.

Figures 6, 7 and 8 were also used to see if the plasmids contained in these organisms were stable over a four month period in $-20^{o}C$ and $-70^{o}C$. These figures showed that at month 0 or before the organisms were stored, and at month 4, all twelve organisms demonstrated plasmid profiles which were not altered. That is the number of plasmids and their size were not altered by storing at $-20^{o}C$ and $-70^{o}C$. These organisms were also tested at months 1, 2 and 3 with identical results to month 0 and 4 (data not shown). The plasmid number and their sizes for the organisms at months 0 and 4 are listed in Table V.

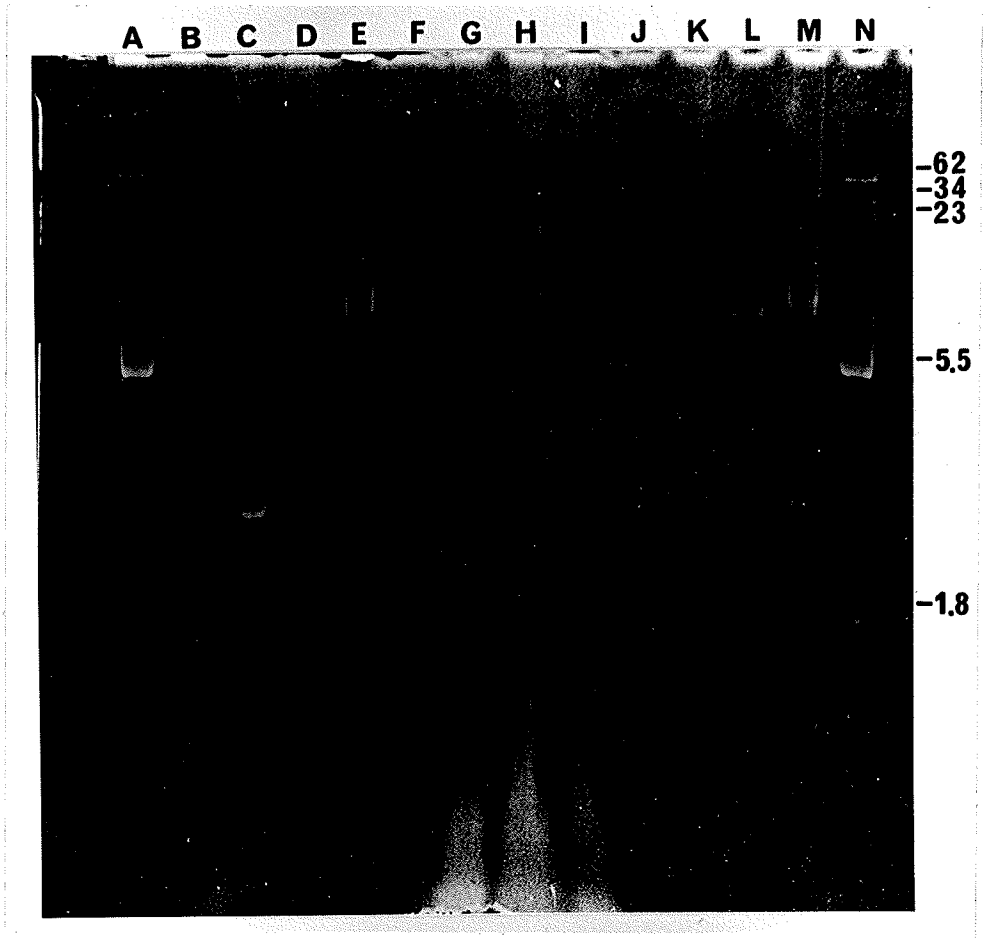

Figure 6: Agarose gel (.7%) of plasmid DNA screened from 12 coagulase-negative staphylococci organisms. A and N. Molecular weight standards, B.7722-1, S. capitis. C and D. 4571 and 4625, S. epidermidis. E and F. 6685 and 6730, S. haemolyticus. G and H. 4595 and 4605, S. hominis. I and J. 5088 and 6007, S. simulans. K and L. 4555 and 7376, S. warneri. M.7092, S. xylosus. These results indicated that lanes B-M respectively contained 1, 2, 2, 0, 0, 1, 1, 1, 1, 0, 0, and 1 plasmids. This was done at month 0, prior to freezing the organisms.

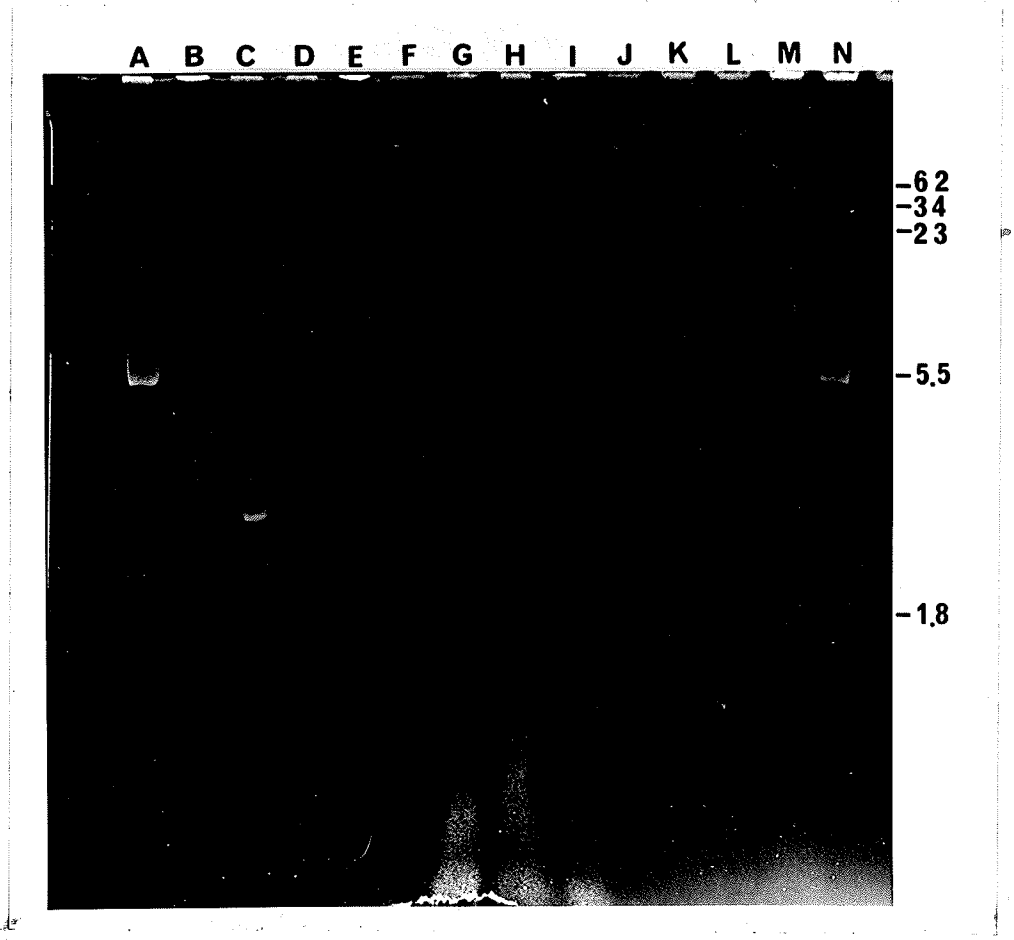

Figure 7: Agarose gel (.7%) of plasmid DNA isolated from 12 coagulase-negative staphylococci organisms which were stored at -20°C for 4 months. These are the same organisms as listed in Figure 6 including the molecular weight standards. The results indicated that lanes B-M respectively contained 1, 2, 2, 0, 0, 1, 1, 1, 1, 0, 0, and 1 plasmids.

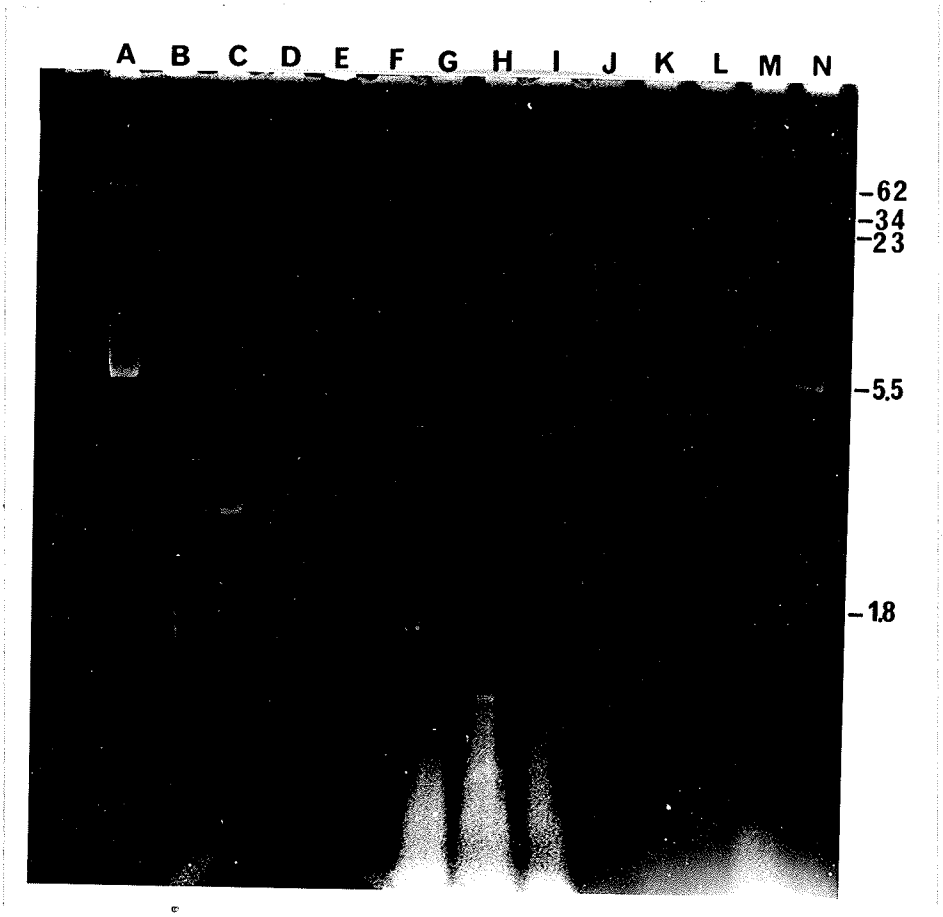

Figure 8: Agarose gel (.7%) of plasmid DNA isolated from 12 coagulase-negative staphylococci organisms which were stored at -70°C for 4 months. These are the same organisms as listed in Figure 6 including the molecular weight standards. The results indicated that lanes B-M respectively contained a, 2, 2, 0, 0, 1, 1, 1, 1, 0, 0, and 1 plasmids.

TABLE V. Plasmid stability

Organism	Month 0 Plasmid No.	Month 0 Plasmid Size(s)	Month 4 Plasmid No.	Month 4 Plasmid Size(s)
7722-1	1	1.8	1	1.8
4571	2	2.4	2	2.4
4625	2	1.8, 20	2	1.8,20
6685	0		0	
6730	0		0	
4595	1	15	1	15
4605	1	15	1	15
5088	1	2.0	1	2.0
6007	1	2.0	1	2.0
4555	0		0	
7376	0		0	
7092	1	2.4	1	2.4

*plasmid size is in megadaltons

C. Common Plasmid Amongst Slime Positive Organisms

When attempting to resolve the problem of the location of the slime producing genes in the coagulase-negative staphylococci the first thing looked at was for the presence of a common plasmid. The plasmid content of 4 slime producing organisms, stock numbers 128, 129b, 4882, and 6081 were looked at for the presence of a common plasmid. This is shown in Figure 9. Lanes A and B contain single plasmids, Lane C has no plasmids, Lane D has 3 plasmids, and Lane E contains 4 plasmids. Lane E contains the standard strain, which is slime negative, obtained from Archer. The only common plasmid amongst these organisms is the lone plasmid from lane B which corresponds to a plasmid in Lane D.

D. Plasmid Curing

Another method used to determine if the slime producing genes were chromosomal or plasmid mediated was curing. By curing or eliminating a lone plasmid from a slime positive organism more evidence could be obtained to determine where these genes are located.

The curing of the lone plasmid from strain 129b is clearly shown in Figure 10. Lanes B and G are separately prepared lysates of the slime producer 129b, and they illustrate that this particular organism contains one plasmid. Lane C is the cured strain of 129b, minus the lone plasmid of molecular weight 5×10^6 daltons. Lanes D, E, and F are subcultures of the cured 129b. This showed that through repeated passage in culture the cured plasmid from 129b does not reappear. This plasmid had been irreversibly lost.

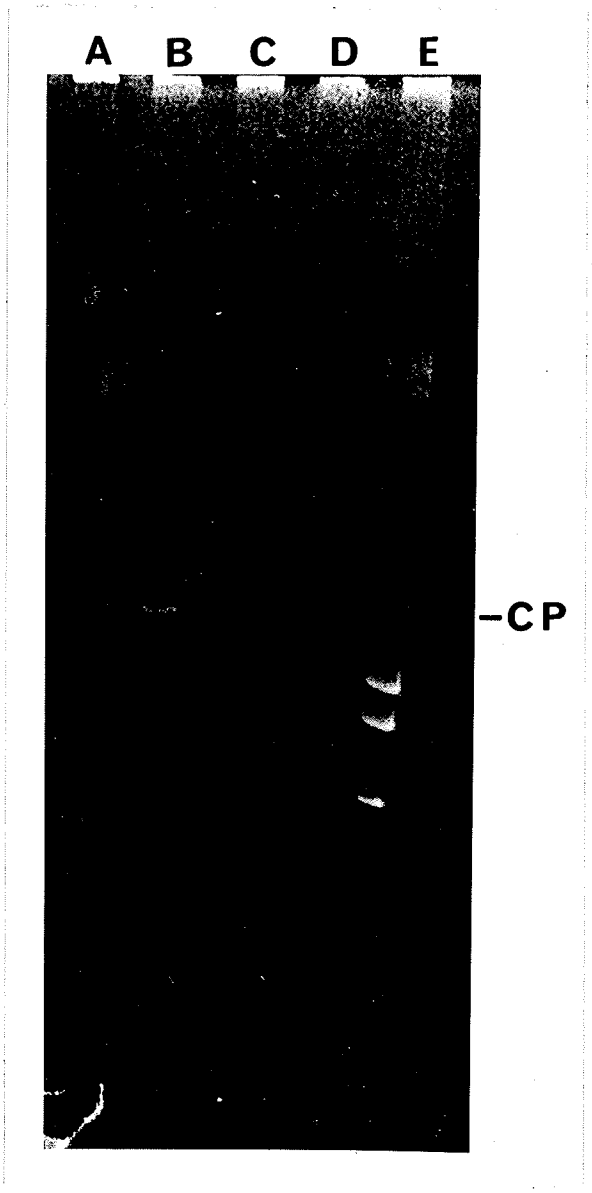

Figure 9: Agarose gel (.7%) of plasmid DNA isolates from 4 slime producing coagulase-negative staphylococci organisms. Lanes A-E respectively contain the lysates of organisms 128, 129b, 4882, 6081 and Archer's standard. There is only a common plasmid in lanes B and D, represented by CP.

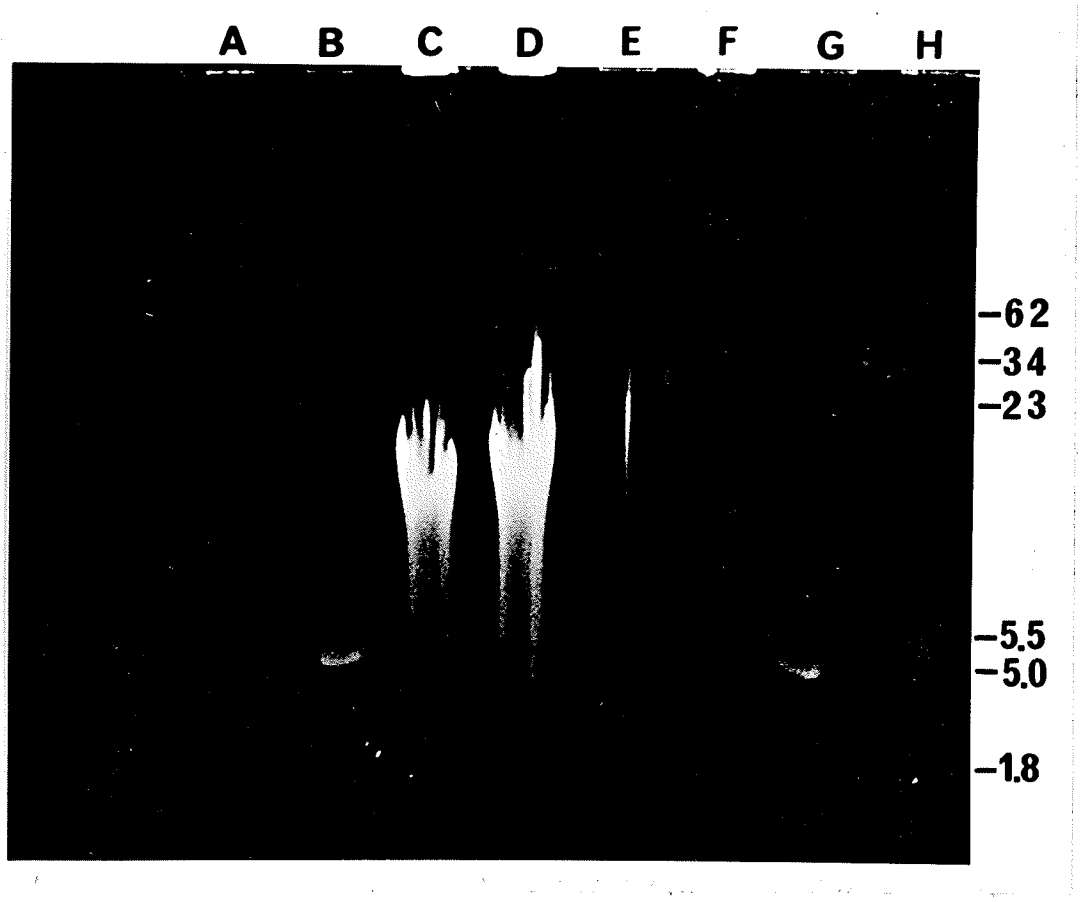

Figure 10: Agarose gel (0.7%) of plasmid DNA isolated from strain number 129b and molecular weight standards. Lanes A and H contain molecular weight standards, lanes B and G contain plsmids from 129b, lane C, is 129b cured, and lanes D, E, and F are subcultures of 129b cured. This illustrates the loss of a 5×10^6 daltons plasmid by 129b.

Subsequent slime tests on all of these organisms, 129b and the four subcultures showed all five of the organisms were slime positive. This indicated that curing of a lone plasmid from a slime positive organism did not eliminate the organism's slime producing ability.

Antibiotic susceptibility tests were done on strain 129b and on the cured 129b strain. It was found that 129b was resistant to penicillin and trimethoprim/sulfamethoxazole (SXT), whereas 129b cured was sensitive to these two drugs. This indicated that the plasmid cured from 129b carried resistance genes to these two drugs.

Api STAPH-IDENT tests were also run on strain 129b and the cured strain. It was found that both of these organisms were S. epidermidis. This indicated that the curing of the plasmid from 129b did not alter its biotype. The only change in the organism, caused by the curing, was the loss of resistance genes to penicillin and SXT.

E. Plasmid Transformation

Another procedure tried in attempting to determine the genetic basis of the slime producing genes in the coagulase-negative staphylococci was transformation. After several attempts it was found that transformation could be achieved in these organisms. We were able to isolate and purify the lone plasmid of strain 129b and transform it into strain 4485. Strain 4485 was a slime negative strain which contained no plasmids. These results are shown in Figure 11. Lanes A and F are the molecular weight markers, lane B is the plasmid from a lysate of 129b, lane C is this same plasmid purified, lane D is a lysate of strain 4485, while lane E is strain 4485 after it had been transformated with the purified plasmid from lane C.

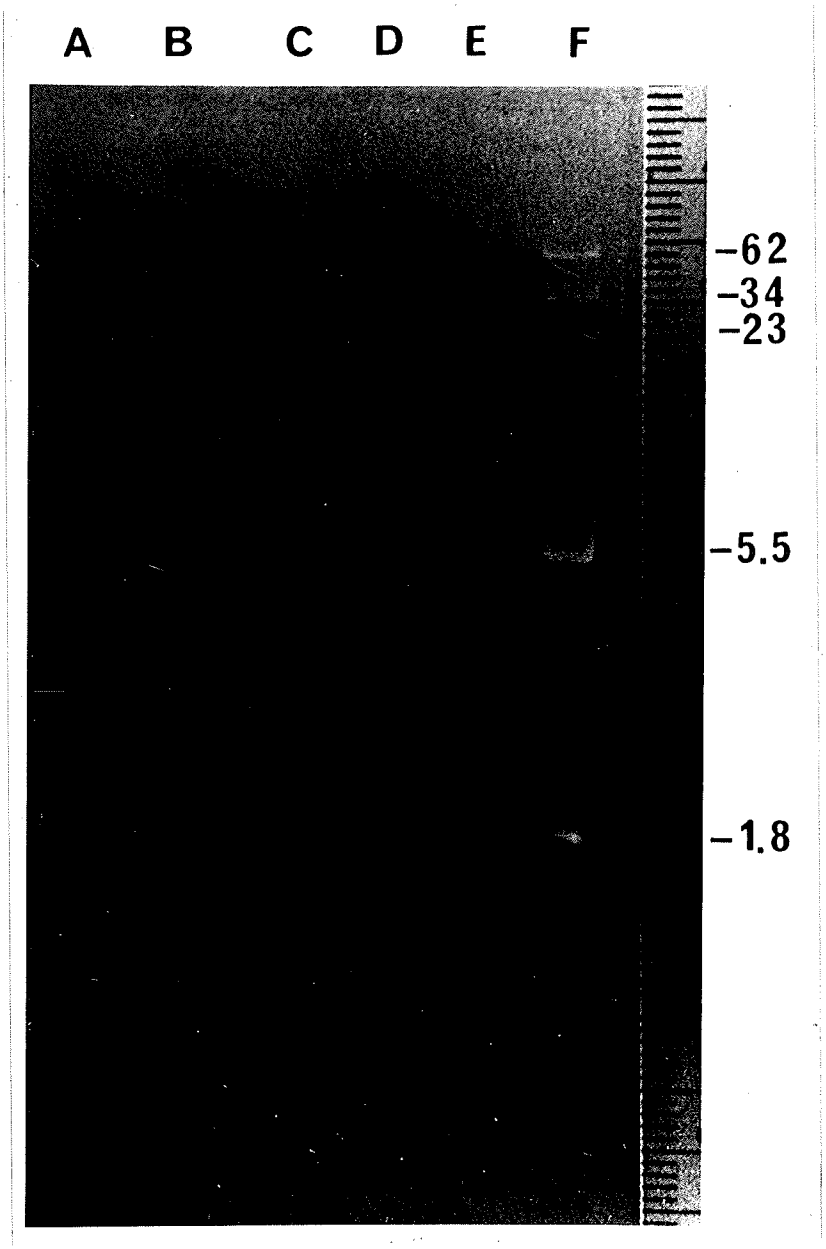

Figure 11: Agarose gel (0.7%) of plasmid DNA isolated from strains 129b and 4485. Lanes A and F represent molecular weight markers. Lane B is plasmid DNA from 129b lysate, lane C is the plasmid from 129b purified, lane D is a lysate of strain 4485, and lane E is the transformed 4485.

This figure shows that the transformation was a success. Following the transformation slime production tests were run on strain 4485, the transformed 4485, and 129b. Strain 129b was found to be positive, while both 4485 and the transformed 4485 were found to be non slime producers. All of this indicates that the slime producing genes from 129b were not transfered to 4485 when the lone plasmid of 129b was transformed into 4485.

Antibiotic susceptibility tests were run on strain 4485 and the transformed 4485. It was known that this plasmid carried resistance genes to penicillin and SXT, while 4485 was sensitive to these two drugs. These tests showed that the transformed 4485 was resistant to penicillin and SXT. Thus the transformed plasmid was being translated in strain 4485.

API STAPH-IDENT tests were also run on strain 4485 and the transformed 4485. It was found that both of these organisms were identified as S. epidermidis. This indicated that the transformation of strain 4485 with the plasmid from 129b did not alter the biotype of 4485. The only change detected in the transformed organism was the gain of resistance genes to penicillin and SXT.

F. Quick Screen for Slime Production

One method used to try and develop a quick screen for slime production by the coagulase-negative staphylococci was biological staining. It was hoped that by incorporating alcian blue or congo red into the growth medium of these organisms it would be possible to distinguish between slime positive and slime negative organisms. Using alcian blue incorporated into the growth medium it was found we were unable to distinguish between slime negative and slime positive colonies. None of the coagulase-negative

staphylococci organisms were affected by the alcian blue. The organisms grew as they would on the growth medium without alcian blue. Using congo red incorporated into the growth medium it was once again found that we were unable to distinguish slime positive from slime negative organisms. Original results using 2 slime positive and 2 slime negative organisms were encouraging. It was found that the two slime negative organisms (129b, and 6113) grew with a dark red pigmentation while the slime positive colonies (820, and 128) grew with a pinkish color. A blind experiment, was then tried, with 10 slime negative and 10 slime positive organisms to see if congo red could differentiate between these two. The organisms were obtained, not knowing if they were slime positive or negative, and grown on the media and then it was determined if they were positive or negative. The results of this test are listed in Table VI. The two columns under actual status are the known slime character of these organisms while the columns under proposed status represents my data from the congo red experiments. Looking at this table, one can see that we were able to accurately predict the character of only half of these organisms, 5 slime positive and 5 slime negatives. The other half we were unable to determine. So this does not appear to be a good method to differentiate between slime positive and slime negative organisms.

The other method used as a possible quick screen for slime negative and slime positive organisms was an antiserum agar technique. In this method the slime was isolated from the rest of the bacteria material, it was purified, used as an antigen in rabbits and the subsequent antibodies were then collected and used to screen for the slime. So basically the results can be divided into 5 sections, 1) isolation, 2) purification, 3) immunization, 4) antibody collection, and 5) screening.

TABLE VI. Congo Red Results

Actual Status		Proposed Status	
Slime (+)	slime (-)	slime (+)	slime (-)
120B	180	130B	7241
129B	61	181B	7722-1
130B	820	120B	820
181B	7722-1	129B	7179
422	6081	180	422
7179	6428	6081	7777
7529	6762	6428	8104
7777	7160	6762	7160
8013	7241	7277	61
8104	7277	7529	8013

In the isolation of the slime, strain 129b a slime positive organism was used. The method employed here, Murthy et al (1983) was originally employed to isolate a capsular polysaccharide from S. aureus. In the isolation of the crude polysaccharide a slightly brownish white material was obtained. This is the same material that Murthy describes in his method. In the purification of the polysaccharide from this crude material Ion Exchange Chromatography was used. The final product was a white fluffy substance with a yield of approximately 25% of the starting crude material. Murthy had a yield of 20%. This material was then analyzed by Thin Layer Chromatography following complete acid hydrolysis of the polysaccharide. The results of this procedure can be seen in Figure 12. Lane A represents mannose, Lane B represents the hydrolysed polysaccharide and Lane C represents glucose. The approximate retardation factor (RF) for each of these were mannose 0.625, hydrolyzed polysaccharide 0.648, and glucose 0.648. This may suggest that the slime polysaccharide is composed of glucose residues. How many glucose residues is not known. The Thin Layer Chromatograph was also analyzed for the presence of amino acids with ninhydrin. This data is not shown because the ninhydrin failed to detect the presence of any amino acids in the purified polysaccharide. Both the hydrolysed and nonhydrolysed polysaccharide were analyzed with ninhydrin.

The nonhydrolysed pure polysaccharide was then used as an immunogen to raise antibodies against the slime of the coagulase-negative staphylococci. The schedule followed here was the one devised by Hurn and Chantler (1980). The blood collected following immunization was separated to give whole serum which was hoped to contain the desired antibodies against the slime polysaccharide. Antiserum agar was prepared using the whole blood sera as well as with the precipitated immunoglobulins of the same sera. In both

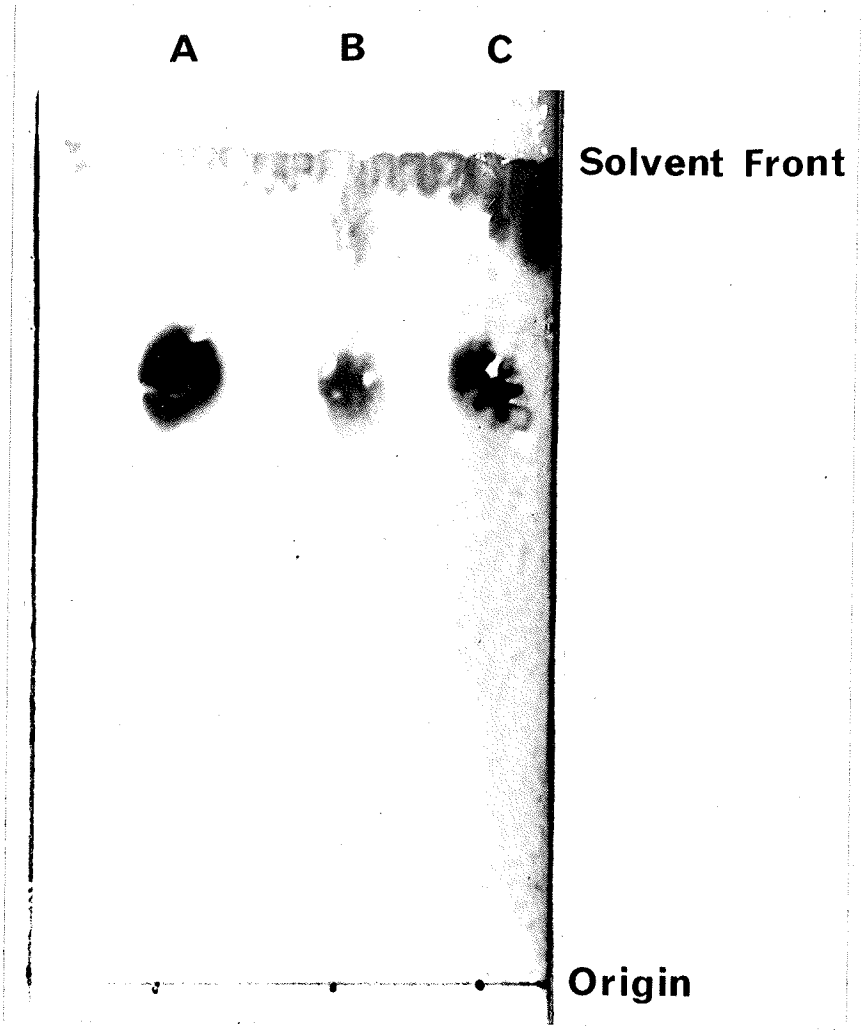

Figure 12: Thin Layer Chromatography analysis of purified polysaccharide. Lane A represents mannose, Lane B represents purified polysaccharide, and Lane C represents glucose. Rf values are Lane A 0.625, Lane B 0.648, and Lane C 0.648. Bials reagent was used to detect the sugars.

cases the results indicated that antibodies to the slime polysaccharide were not raised. That is, when slime positive colonies were grown on the antiserum agar plates no halo of precipitation formed around the colony. The slime positive colonies grew the same as slime negative colonies on these plates. These two could only be differentiated by examining them for slime production using Christensen's (1981) method. This was disappointing data when compared to Bradshaw et al (1980) and, Michaels and Stonebraker (1975) data in which they were able to differentiate organisms due to the formation of a halo of precipitation.

Gel diffusion was also used to check for the formation of antibodies against the slime polysaccharide. In this case the whole sera and precipitated immunoglobulins were tested against the immunogen of isolated polysaccharide rather than against whole organisms. This method failed to indicate the presence of any antibodies against the immunogen of pure polysaccharide. If antibodies had been present a line of precipitation would have formed between the sera and the polysaccharide.

DISCUSSION

A. Introduction

The purpose of these studies on the coagulase-negative staphylococci were threefold. 1) We have attempted to develop an accurate plasmid profiling technique to study the plasmids of these bacteria. 2) We have attempted to determine the genetic basis of slime production by these bacteria and; 3) We hoped to find a quick screening method to differentiate between slime positive and slime negative organisms.

B. Plasmid Profiling

1. Methodology

Within the last few years the molecular analysis of plasmids has been applied very productively to the investigation of various microorganisms. There are basically two molecular techniques useful in plasmid analysis, direct and indirect techniques. Direct methods involve hybridization of DNA strands from different sources which may allow a quantitative assessment of base sequence homology. Indirect methods include agarose gel electrophoresis and restriction endonuclease analysis. Electrophoresis of plasmids in an agarose gel enables one to estimate the size of a plasmid, and to prove the presence of similar bacteria between different isolates (Farrar, 1983).

In attempts to develop a plasmid profiling technique to investigate the coagulase negative staphylococci several methods were experimented with

before settling on one. All of the methods experimented with ultimately proved to be inadequate in some aspect. Some of these methods and their inadequacies are described here. Forbes and Schaberg (1983) developed a plasmid isolation technique for S. epidermidis to study conjugation. This method was used on 6 coagulase negative staphylococci strains obtained from St. Boniface Hospital. It was applied four times over a period of seven days. Results from these experiments gave inconsistent plasmid numbers. Also there was excessive contamination from chromosomal DNA material and plasmid artifacts, i.e. open circle plasmids. Another method, employed by Wilson et al (1978), was also found to be inadequate. This method was a rapid procedure for the detection of plasmids in S. epidermidis strains. This method was found to give inconsistent analysis of bacterial strains. That is, some strains were lysed while others were not. Also this method gave excessive contamination with chromosomal DNA, and open circle plasmids, for the strains that were lysed. One more method tried was provided by L. Slaney of the department of Medical Microbiology, U. of Manitoba. This was a three day method to isolate plasmids from S. epidermidis strains. The results obtained from this method were consistent in the numbers of plasmids recovered, however once again there was considerable contamination with chromosomal DNA and plasmid artifacts. The biggest disadvantage of this method was that it took three days to obtain proper results. A good typing scheme should give results in less time than this. Another method experimented with was published by Kloos (1980). The results from this method were fairly consistent, however some strains of coagulase-negative staphylococci were not lysed sufficiently to give any results. Once again there was also contamination with chromosomal DNA and plasmid artifacts. So this method was deemed nonsuitable for our purposes.

We also experimented with a rapid boiling method published by Holmes and Quigley (1981). The major difference in this method was that it employed the use of horizontal gel apparatus, at a much lower voltage (3V) for a much longer time (12 hrs) than used for vertical gels. The results of experiments here were poor. There was insufficient lysis of the organisms to be able to detect the plasmids in the gel. However some chromosomal DNA was detected in the gels. One other method experimented with was a staphylococcal plasmid screen developed by Cooksey (1981). This method seemed to give good consistent results, however like most of the others, the results were contaminated with chromosomal DNA and plasmid artifacts. See Figure 2.

After trying all of these methods, it was decided to take one method, and work with it until the results became satisfactory for our needs. It seemed that major difficulties to overcome would be to obtain consistent lysis, and to get rid of chromosomal DNA and plasmid artifacts contamination. The method decided on was the staphylococcal plasmid screen developed by Cooksey (1981). The first problem faced was getting consistent lysis for all coagulase-negative staphylococcal tested with this method. The first thing tried, to improve lysis, was fast freezing at $-70^{o}C$, and thawing of the bacteria prior to lysostaphin treatment. Freezing and thawing should weaken the bacterial cell wall thus making it more susceptible to lysis. After examining the cells, using the gram stain procedure and a light microscope before and after freezing and thawing no difference in the bacterial cells was found. As well it was found that this method did not enhance the lysis of the bacteria after lysostaphin treatment. The next step used to try and remedy this situation was to change the enzyme used for lysis. This method used lysostaphin and we

tried using mutanolysin (Sigma) instead. Various concentrations of this enzyme were tried, and it was found that even when large amounts of it were used the lysis of the bacteria was minimal. Therefore this enzyme was unacceptable for our purposes. At this point we turned our attention back to the use of lysostaphin. Cooksey's method used 30 ul of a 1 mg lysostaphin per ml of TES buffer, for 20 minutes in a 37°C water bath. Therefore it was assumed that by increasing either the concentration of lysotaphin or by increasing the incubation period lysis of the organisms could be enhanced. Both factors were experimented with extensively before realizing the goal of consistent lysis. To obtain consistent lysis it was determined that 50 ul of a 1 mg lysostaphin per 1 ml TES buffer should be incubated with the organisms for 30 minutes in a 37°C water bath. To arrive at these figures incubation time was varied from 20 minutes to 12 hours, and lysostaphin concentration was varied from 30 ul to 200 ul. It was also found that by using too much lysostaphin (>100 ul) or by incubating too long (>1 hr) there was too much lysis of the organisms. This resulted in extensive chromosomal contamination. Another factor which was altered to enhance lysis was the amount of TES buffer containing 8% sodium dodecyl sulfate (SDS) added to the samples following incubation. Cooksey added 250 ul of this solution to each sample. It was found that by adding 400 ul of this solution results were improved. That is lysis was more consistent. So consistent lysis was achieved by increasing lysostaphin concentration, incubation time, and the amount of 8% SDS added.

Having solved the lysis problem, we now focused our attention on the problem of eliminating chromosomal DNA, and plasmid artifact contamination. Cooksey's method, as it was, contained a single step to eliminate this type of contamination. This was the treatment of the lysed cells with a 65°C

water bath for 10 minutes. This treatment was found to be inadequate because the contamination was still very heavy. It was found that by increasing the incubation period to 20 minutes, the contamination was lessened, but still represented a problem. Thus another step was needed to give lysates clear of chromosomal DNA and plasmid artifacts. The literature contains many other plasmid analysis methods which contain procedures to eliminate such a problem. These procedures all attempt to eliminate the linear chromosomal DNA and the open circle plasmids, while leaving the closed circular DNA intact. Some of these procedures which were tried with limited success were boiling, sodium chloride treatment, and phenol treatment. Each of these procedures was applied following the 68^{o}C water bath treatment. Having no success with these treatments it was decided to try an alkaline procedure described by Vinograd and Lebowitz (1966). This procedure raised the pH of the DNA containing sample, such that all of the double stranded DNA became unstable and separated. However the closed circular plasmids remained together because they were intertwined. Now when the pH was lowered to 7, the closed circular plasmids reannealed, whereas the chromosomal DNA and open circle plasmids did not reanneal. This procedure cleared up almost all of the contamination in the samples. This is illustrated in Figure 3.

Another procedure tried to add further clarity to the samples, was treatment of the samples with RNAase. After applying this procedure no difference was found in the results. This was because the RNA ran at the bottom of samples, causing no interference with the DNA bands.

A final alteration to Cooksey's method was made to the media the coagulase-negative staphylococci were grown on. It was believed that to enhance the susceptibility of the coagulase-negative staphylococci to

lysostaphin the organisms should be grown on Tryptic Soy Agar containing 0.3% yeast extract (Sigma) and 0.5% glycine (Sigma). This medium prevents incorporation of serine into the cell wall which decreases sensitivity to lysostaphin. It was found that cells grown on regular sheep blood agar plates were just as sensitive to lysostaphin as were cells grown on the special supplemented agar. Thus a special medium was not required in order to get satisfactory results.

All of the alterations made from the original CDC method are listed in Table II. The final CDC method employed in our experimental work is listed in Table III, along with the reasoning for each major step. Figure 4 illustrates the good consistent results that were obtained using the revised CDC method.

2. Interpretation of DNA Bands

After looking at the resulting picture from an agarose gel electrophoresis run it is not always easy to interpret the results. There could be bands in the picture representing closed circular plasmid DNA, plasmid artifacts, RNA, or chromosomal DNA. The lysate procedure should get rid of most of the chromosomal DNA and plasmid artifacts, however they are usually still visible in the picture. According to Aaij and Borst (1971) these different types of DNA and RNA are easy to identify by the bands themselves. The RNA band will invariably travel with the leading edge of the tracking dye, and can be seen in the photograph at the bottom of the gel. If one desires the RNA bands can be completely eliminated by treating the lysate with RNAase. I found that this was not necessary because the RNA bands did not interfere with picture clarity in any way.

This was shown by running the same organisms with and without RNAase and comparing pictures of the gels after electrophoresis, data not shown. In other plasmid analysis procedures some investigators used RNAase while many others did not find its use a necessary procedure. Forbes (1983), Holmes and Quigley (1981), and Kloos et al (1980) all used RNAase in their plasmid isolation procedures, while Wilson et al (1978), Bunkle and Sippel (1984), and Kato and Liu (1981) did not include the RNAase step. So plasmid profiling can be successful with or without an RNAase step. The bands representing open circular plasmid DNA and closed circular plasmid DNA are differentiated as follows. The closed circular band is very definite and it should be a flat band or a band with its ends turned slightly upward. The open circle plasmid band is usually quite faint and it is a band with its ends turned downwards. There are examples of this in Figure 5. When looking at an agarose gel electrophoresis picture, one can easily distinguish between chromosomal DNA and plasmid DNA. Plasmids give you thin definite bands, whereas chromosomal DNA gives a thick blurry band which will be common to all organisms in a given genera. Results will always be better when this chromosomal DNA band has been reduced to almost nothing. This prevents the chromosomal DNA band from masking the presence of a plasmid DNA band. So ideally an agarose gel electrophoresis picture should contain only closed circular plasmid bands. However in most instances contamination with open circular plasmids and chromosomal DNA can not be completely eliminated. Thus one must be able to interpret these results to differentiate between closed circular plasmid DNA and contaminating DNA.

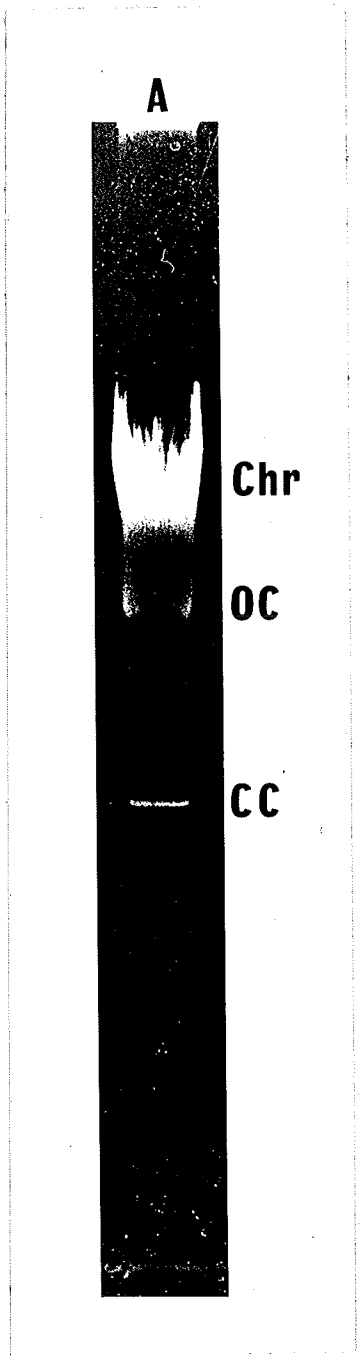

Figure 5: Agarose gel (0.7%) of plasmid DNA isolated from strain 6008. Lane A represents the lysate which illustrates a chromosomal DNA band (Chr), an open circle plsmid DNA band (OC), and a closed circle plasmid DNA band (cc).

3. Application to Staphylococcus species.

When one is attempting to apply a plasmid analysis procedure to investigate organisms, these organisms must consistently contain plasmid molecules in order for the procedure to be applicable. Organisms which lack plasmids can not be typed using plasmid profiling. Kloos et al (1980) did an extensive study on the plasmid composition of Staphylococcus species. This group looked at a total of 342 staphylococci representing the then 13 recognized species, and found that almost all of these contained at least 1 plasmid and the majority of these strains demonstrated the presence of 2 or more plasmids. The only human species which Kloos did not look at was S. auricularis. My results supported the theory that the majority of these organisms do in fact contain plasmids. The fact that 25% of my organisms do not contain plasmids is hardly significant, since only 12 organisms were studied (Figures 6,7 and 8). In a much larger sample this percentage would surely drop. In Kloos's sample 89% of the coagulase-negative staphylococci organisms contained plasmids.

4. Is This An Ideal Typing Method?

When designing a typing technique such as plasmid profiling one must be aware of the properties and characteristics of an ideal typing system According to Aber and Mackel (1981) there are eight of these characteristics. A useful and effective typing system should be standardized, reproducible, sensitive, stable, readily available, inexpensive, widely applicable, and field tested in conjunction with epidermiologic investigation. It was our objective through experimentation

to prove that the method developed to investigate the coagulase-negative staphylococci met these criteria, and was in fact a useful and effective typing system.

This method was very affordable, and readily available. The method employed chemicals which were relatively inexpensive and available in almost all labs. The only big expense incurred was the purchasing of gel electrophoresis apparatus. Most labs already possess such equipment thus they will not have to make this major purchase.

It was also shown that this method was broadly applicable to all microorganisms of interest. These organisms were the ten recognized human species of the coagulase-negative staphylococci. Twelve coagulase-negative staphylococci strains were received from St. Boniface Hospital representing 7 of the ten human species. All ten species were not studied because S. cohnii, S. saprophyticus, and S. auricularis species were not available for investigation. As seen in Figures 6,7 and 8 this method was able to effectively give a plasmid profile for each strain studied in each species of the coagulase-negative staphylococci. It was also observed that some methods were unable to lyse certain coagulase-negative staphylococci strains. However, the method eventually settled on was able to lyse, and give an accurate plasmid profile for each coagulase-negative strain tested. So this method satisfied the criteria of reproducibility.

A good typing system should also be sensitive enough to distinguish microorganisms which are biologically or genetically similar but not identical. Examples of this have been cited in the literature review where researchers have used plasmid profiling to distinguish between seemingly identical organisms. Perhaps the best example pertaining to the coagulase-negative staphylococci was work done by Parisi and Hecht (1980).

They took coagulase-negative staphylococci organisms which were found to be identical by conventional typing methods, and proved these organisms to be nonidentical using plasmid profiling. Even though these organisms had identical biotypes, and phage types, their plasmid profiles were found to be vastly different. Organisms which appeared to be identical were in fact nonidentical. Although experiments in this area were not performed we feel our plasmid profiling technique still did meet this criteria. The fact that this method was able to accurately give plasmid profiles of all coagulase-negative staphylococci organisms studied suggested that if organisms such as the one Parisi and Hecht studied were encountered this method could differentiate between them. This method had satisfied another of the criteria of an ideal typing method.

This method also satisfied the criteria of standardization. A coagulase-negative staphylococci strain was obtained from Archer, which contained a known number of plasmids. This strain contained five plasmids, some of high molecular weight and some of low molecular weight. When this strain was tested using our plasmid profiling technique all of the known plasmids were recovered (Figure 9, Lane E). It was felt that this proved the standardization of this method especially since the standard strain of Archers' contained plasmids ranging from high molecular weights to ones with low molecular weights. Thus this method was capable of detecting a broad range of plasmids.

A typing system should also be readily reproducible and stable over time. By being reproducible Aber and Mackel (1981) mean that the method should be defined, such that it can be easily reproduced by other investigators with similar results. By being stable over time, Aber and Mackel (1981) mean that the system should be based upon a stable genetic

property. This method satisfied both of these criteria. This can be seen by looking at the results from Table V in which the stability of the plasmids, at -20°C and -70°C was tested. First this method was used each time a gel was run of the 12 coagulase-negative staphylococci tested for plasmid stability. It was found that this method and the results were both easily reproduced by following the methodology that was developed. By looking at Table V and Figures 6,7 and 8 where the plasmid stability results are listed one can see this. The fact that the Figures consistently contained the same number and size of plasmids indicated that the method was reproducible.

In nature plasmids are not stable genetic properties of microorganisms. They are continuously being transferred between and lost from bacterial species. These mechanisms of plasmid DNA transfer include transduction, transformation, conjugation, and mutation. Even though plasmids are not stable in their natural environment, it was shown that the plasmids of the coagulase-negative staphylococci were stable under artificial conditions, and can be used to type these organisms. These plasmids were found to be stable over a period of four months when they were stored at both -20°C and -70°C. This is illustrated by Figures 6,7 and 8 and Table V. Thus it is possible to study the spread of endemic plasmids in these organisms, and these organisms can be stored indefinitely without fear of losing the specific plasmid from the bacterial strains. So one more of the criteria of the ideal typing system was satisfied.

The final criteria for an ideal typing system was that the method should be field tested in conjunction with epidemiologic investigation. Actual epidemiologic investigations with this method were not done however experiments that were done suggest that this method is a good one. These

experiments were the plasmid stability ones and the ones showing that all coagulase-negative staphylococci species can be analysed in this way. These experiments suggested that the criteria for field testing had been satisfied.

This method for the plasmid profiling of the coagulase-negative staphylococci appears to satisfy the eight criteria of a useful and effective typing system suggested by Aber and Mackel (1981).

5. Plasmid Stability in These Organisms

In Figures 6,7 and 8 and Table V, it was shown that when a group of twelve organisms representing several of the coagulase-negative staphylococci species were stored for an extended period of the time their plasmid content remained stable. These organisms were stored for a period of four months at both $-20^{o}C$ and $-70^{o}C$. This was an important observation because these bacteria and their plasmids must often be stored for extended periods of time when doing epidemiologic studies. Lyon et al (1982) did a study on nosocomial strains of S. aureus and studied organisms which had been stored for up to five years. Markowitz et al (1983) studied the plasmids in Enterobacter cloacae and studied organisms which had been stored for up to seven years. By showing that these organisms and their plasmids can be safely stored at $-20^{o}C$ or $-70^{o}C$, it was shown that long range epidemiologic investigations need not worry about plasmid stability. That is plasmids which were present five years ago will still be present in the organism now, after being adequately stored at $-20^{o}C$ or $-70^{o}C$.

C. Genetic Basis of Slime Production

Slime production by some coagulase-negative staphylococci strains may be an important virulence marker. Although not much is known about the virulence of slime production, some investigators have made a direct correlation between it and virulence of the coagulase-negative staphylococci. Christensen et al (1982) showed that 67% of clinical coagulase-negative staphylococci isolates produce slime while only 37% of randomly collected blood culture contaminants and skin isolates produced slime. Yoshida and Ichiman (1981) studied the virulence of the coagulase negative staphylococci in mice. They observed that the most virulent strains were those which were encapsulated or produced slime. Christensen et al (1983) looked at clinical strains of coagulase-negative staphylococci from patients with catheter associated sepsis. They found that strains which produced slime were more virulent than strains which did not. They also observed that a higher, although not significant, proportion of resistant strains produced slime.

Although the structure of the slime remains unknown, previous work by Christensen et al (1982) suggested it is composed of polysaccharide material. They found that this material stains with alcian blue, and has nutritional requirements for autoclaved dextrose and tryptone. This suggested that it is made up from a carbon fragment and an amino acid.

The stability of the slime characteristic for these coagulase-negative staphylococci also remains a mystery. Once again previous work by Christensen et al (1982) suggested that this is a stable characteristic. This group suggested that the slime characteristic may be turned on or off in a manner analogous to rough-smooth strains of pneumococci or

fimbrate-nonfimbrate strains of E. coli. They also speculated that this may be a relative phenomenon rather than a simple positive or negative.

Since it appears that slime production by the coagulase-negative staphylococci is an important virulence marker, it would be important to determine whether the slime producing genes were chromosomal or plasmid mediated. This was important because if the genes were chromosomal mediated they would be stable, and would not be transferred between the Staphylococcus strains. However, if the genes were plasmid mediated then they would be unstable and could easily be transferred between these organisms. They could be transferred via conjugation, transduction or transformation.

The first thing we decided to look at was the stability of slime production in the coagulase-negative staphylococci. Christensen et al (1982) observed that this was a stable characteristic through animal or extended lab passage. This meant that upon successive subcultures or animal passage these organisms ability to produce slime did not change. This group also found that they were unable to manipulate cultural conditions to encourage slime production by a previously nonadherent strain. So they were unable to alter an organisms ability to produce slime or not. Christensen et al (1983) looked at the characterization of clinically significant strains of coagulase-negative staphylococci. In this study they found slime production in all staphylococcal species except S. warneri and this production appeared to be stable. The absence or presence of slime production was constant for strains isolated from multiple blood cultures obtained on the same day or different days. So all indications from this early work by Christensen indicated that slime production was indeed a stable characteristic indicating it was chromosomal

mediated. However, a study by Low et al (1985) on the reproducibility of a test for slime production indicated that this might not be such a stable characteristic. Although their results have not been completely analyzed, the statistical significance has not been determined, it appeared that slime production was not a stable characteristic. Their results showed that the slime production test was not observer variable but was interest variable. They also found that freezing at $-70^{o}C$ resulted in a correlation of 84% for slime production before and after testing. So this indicated that slime production may not be as stable as previously believed. Also, these results did not clarify whether this characteristic was chromosomal or plasmid mediated. Early indications were that it was chromosomal, however the work by Low et al indicated that this may not be true.

The next thing looked at to determine the genetic basis of slime production in the coagulase-negative staphylococci was the presence of a common plasmid among slime producing organisms. Four coagulase-negative staphylococci organisms were received from St. Boniface Hospital which were previously found to be slime positive, B-lactamase negative, and sensitive to all antibiotics they were tested against. These organisms were chosen because it was believed that they would contain few plasmids, since antibiotic resistance genes are usually contained within plasmid DNA. These organisms were analyzed using plasmid profiling to see if there was a common plasmid. The results, shown in Figure 9, indicated that these four organisms did not contain a common plasmid. This supported the idea that slime production was chromosomal mediated. It could also be possible for slime genes to be on more than one plasmid, thus eliminating the need for a common plasmid in slime positive bacteria. However, the fact that one of the slime positive bacteria studied here contained zero plasmids ruled out

this possibility. The fact that the four slime positive coagulase-negative staphylococci did not contain a common plasmid, and one of these four contained zero plasmids, was strong evidence for slime production being chromosomal mediated.

The next experiment that was used to determine the genetic basis of slime production was the curing or elimination of plasmid DNA from a coagulase-negative staphylococcal organism. In these experiments a slime positive coagulase-negative staphylococci which contained a single plasmid was used. This particular strain was chosen from the organisms used to check for a common plasmid. The reasoning here was that if the lone plasmid from this organism was cured strong evidence to help characterize these slime producing genes would be obtained. If the cured strain remained slime positive this would be evidence for chromosomal mediation, but if the organism became slime negative, this would be evidence for plasmid mediation. When doing these experiments it was hard to find cured bacteria. There was no way to select for them, because this strain was supposedly sensitive to all antibiotics. Therefore to determine if an organism had been cured by the ethidium bromide it had to be grown and its plasmid content checked to see if the plasmid had been cured. After finally curing a bacterial cell of this particular plasmid, Figure 10, antimicrobial sensitivities were run of the strain with the plasmid, and without it. It was at this point that it was discovered that prior to curing this organism was resistant to penicillin and SXT. After curing this organism became sensitive to both penicillin and SXT. Thus the plasmid carried genes which coded for resistance to these two antibiotics. If this had been known earlier it would have been much easier to find a

cured organism. The cured cells could have been selected by growing a number of ethidium bromide treated cells on a regular blood agar plate, and then replicate plate them onto a selective media containing the penicillin and SXT. The colonies which did not grow on the replicate plate would represent the organisms which had been cured of the plasmid. This could then be verified by checking the organisms plasmid content via plasmid profiling.

After successfully curing the lone plasmid from this slime positive strain the plasmid-less strain was checked to see if it had remained slime positive or if it had become slime negative. The cured strain remained slime positive, suggesting that the slime producing genes were not contained in the plasmid DNA. If the genes were in the plasmid DNA, the slime producing ability would have been lost when the plasmid was cured. Although this evidence could not be called conclusive, I do feel it was solid evidence suggesting chromosomal mediation.

The one difficulty with the curing evidence was the possibility that the slime producing gene could be situated within a transposable genetic element. As reported by Cohen and Shapiro (1980) transposable genetic elements or transposons are pieces of DNA which can be transferred from one molecule to another, between plasmid and chromosome or plasmid to plasmid. The result of the transposition process was that a segment of DNA originally present on one molecule was transferred to a different molecule that has no genetic homology with the transposable element or with the donor DNA. The end result of all this was that now the donor DNA and the receiving DNA each had a copy of the transposon DNA.

Even though the lone plasmid from this coagulase-negative staphylococci strain had been cured and the strain remained slime positive, it could

still be possible that the slime producing genes were on the plasmid. The genes could be situated in both the plasmid and chromosomal DNA, and when the plasmid was cured the chromosomal genes continued to produce slime. A way to determine if this was the case was the transformation of the lone plasmid from this strain into a slime negative strain.

Transformation refers to the ability of bacteria to acquire a heritable altered phenotype by the acquisition of DNA from an external medium. DNA taken up by this process could be in the form of chromosomal fragments or plasmid DNA. In this case the lone plasmid from the coagulase-negative staphylococci slime positive strain was isolated and then transformed it into a coagulase-negative staphylococci slime negative strain which contained zero plasmids. This is shown in Figure 11. If this plasmid contained the slime producing genes, then theoretically it should have transformed the slime negative strain into a slime positive strain. It was known that the transformation process did work because it was selected for after the experimental procedure. The slime positive strains plasmid carried resistance genes for penicillin and SXT, while the slime-negative strain was sensitive to these two antibiotics. Therefore if the transformation was a success then the slime negative strain would become resistant to these two antibiotics. A slime-negative colony was recovered from the selection plate and to be sure that it had received this plasmid it was analyzed via plasmid profiling.

It was found that the plasmid was present in the slime negative strain which indicated that the transformation had worked. The slime producing ability of this transformed slime-negative strain was now investigated. All three strains, slime positive, slime negative, and transformed slime negative were tested. It was found that the slime positive remained

positive, the slime negative remained negative and the transformed slime negative remained negative. Observing that the transformed plasmid was being translated because the resistance genes of the plasmid were active indicated that the transformed plasmid did not carry genes which encoded for slime production. This was strong evidence suggesting that the slime producing genes for coagulase-negative staphylococci are situated within the chromosomal DNA.

All of this evidence together strongly suggested that the slime producing genes are chromosomal mediated. However, to be sure of this one would have to map the chromosomal DNA of the Staphylococcus, isolate the slime producing gene, and then clone it into a suitable vector.

D. Quick Screen for Slime Production

The method presently used to detect slime production in a coagulase-negative staphylococci strain is a rather time consuming process. This method published by Christensen et al (1982) requires incubation for 24 hours, and a separate test is required for each colony to be tested. A method such as this is accurate however it cannot be classified as quick. Therefore it would be worthwhile to develop a quick screen method to determine if a colony produced slime. The two methods used to develop a quick screen for slime production by the coagulase-negative staphylococci were the use of congo red and alcian blue and the use of antiserum agar. These methods required overnight incubation of the colonies being tested. However they were capable of screening many colonies at the same time.

Congo red is a biological stain certified by the Biological Stain Commission for use with Mayer's hemalum as a cytoplasmic stain on tissues

fixed in Bouin's fluid, also as a stain for amyloid in Highman's technique. This stain was used by Dr. R.C. Miller (Department of Microbiology, University British Columbia, Vancouver,B.C.) in his work on the molecular cloning of the cellulase gene. Miller observed that this dye was very efficient in its ability to stain polysaccharide material. Alcian blue is a biological stain certified by the Biological Stain Commission for the staining of polysaccharides. Christensen et al (1982) observed that slime material stained with alcian blue which suggested that slime is of a polysaccharide nature. Although precise characterization of the slime structure awaits biochemical analysis, the nutritional requirements for autoclaved dextrose and typtone suggest that it is made up from a carbon fragment and an amino acid. The amino acid could be glutamine a rich component of casein. Knowing all of this it was decided to try using these two biological stains incorporated into growth medium to screen for slime positive colonies. It was hoped that the bacteria growing on the surface of the agar plates would become selectively stained. The slime positive colonies would stain, while the slime negative ones would not.

The results using alcian blue incorporated into the culture media were disappointing. It was observed that both the slime negative and slime positive colonies failed to pick up the dye. Although the reason why these colonies were not stained by alcian blue is unknown, it could be possible that the dye became bound to the glucose in the medium and was unavilable to stain the bacteria. A higher concentration of dye was tried which did not improve the results. Since alcian blue was a proven stainer of polysaccharide material there must have been an inability on the dyes part to escape the medium and work under such conditions. Since the bacteria are surrounded by peptidoglycan which contains polysaccharide material, and

if the dye was available then at least this polysaccharide material should stain.

The results using congo red were better, but not good enough to use as a slime screening test. The congo red was able to stain the bacterial colonies, however it stained both the slime negative and slime positive colonies. Using a small group of strains at first it was possible to distinguish between slime negative and slime positive colonies. However, when a blind experiment using twenty different strains was ran, it was not possible to distinguish between slime positive and negative colonies. This is shown in Table VI. The congo red had probably stained the polysaccharides of the staphylococcal cell walls plus the slime of the slime positive colonies. However there was such a small amount of slime present that its staining did not stand out amongst all of the other polysaccharide in the cell walls. So congo red staining could not be used for slime production screening. Ideally, to be able to use a method such as this to screen for slime production, a biological stain that will specificially stain the slime was needed. However, before this could be done the structure of the slime would have to be determined.

If in fact the colonies that produce slime manufacture too little of it to detect by staining then a method was required to detect minute amounts of slime. It was decided that the best direction to follow here would be the antibody-antigen reaction. Antiserum agar had been used frequently and successfully to identify strains of bacteria which possessed a unique exopolysaccharide such as the slime of the coagulase-negative staphylococci. Bradshaw et al (1971) used this method to identify bacterial antigens which were cross-reactive with the capsular polysaccharide of Haemophilus influenzae type b. West et al (1985) used

antiserum agar for identification of Smith type exopolysaccharides in clinical isolates of Staphylococcus aureus. Based on the success of these and other researchers we proposed to isolate the slime, raise antibodies to it, and then use these antibodies incorporated into agar to screen for slime production. The end result of all of this was that the desired results of halo precipitation around slime producing colonies was not achieved. Once again the exact reason was unknown however there is room for speculation as to why this procedure failed to work as we had hoped it would. It could be possible that the slime was never isolated prior to immunization. However, analysis of the material that we did isolate suggested that this was in fact of a polysaccharide nature. This material seemed to be made up of a single sugar residue, as shown in Figure 12. This fits with what other investigaters such as Christensen et al (1982) speculated about the structure of the slime material. Perhaps inadvertently the rabbits were made tolerant to the antigen. However, since a potent adjuvant such as Freund's complete adjuvant was used it was extremely unlikely that tolerance occurred. It could also be argued that perhaps rabbits are not suitable animals for producing these antibodies. West et al (1985) used rabbits to raise antibodies in their antiserum agar work. Bradshaw et al (1971) and Schneerson et al (1972) also used rabbits, while Kabat (1968) observed that rabbits were nonimmunogenic when immunized with dextran material. If we assume no errors were made in the methodologies applied, it would seem that the slime material was a poor immunogen. Perhaps the molecules in their natural form are too small or lack the tertiary structure necessary to serve as efficient immunogens. These molecules could also have been degraded in the tissues and circulation by specific enzymes and by nonspecific proteases before they

contacted immunocompetent cells. To improve the immunogenic capabilities of the slime molecules one could couple them to large suitable carrier molecules or perhaps manipulate them, prior to immunization, to get dimers or polymers of the molecules.

If this procedure had worked it could have been used for screening for slime positive colonies. It could also have been used to determine if the slime genes are on the chromosomal DNA, and their approximate position on the staphylococcal gene map. This could have been achieved by using mutagenesis procedures, or by transforming chromosomal DNA between non slime and slime producers.

SUMMARY AND CONCLUSIONS

This investigation has provided insight into the problems of developing an ideal typing system. It has also provided additional evidence for chromosomal mediation of the slime producing genes in the coagulase-negative staphylococci. Finally it has indicated that the slime molecules, isolated from a coagulase-negative staphylococci may not be a good immunogen. Several conclusions were reached in this study.

1. Many of the published plasmid profiling techniques are inconsistent in their performances.
2. The CDC method proved to be a satisfactory technique following a few alterations.
3. The altered CDC method does satisfy the 8 criteria of an ideal typing system.
4. The plasmids of the coagulase-negative staphylococci are stable at $-20^{o}C$, and $-70^{o}C$ over a 4 month period.
5. Slime production by the coagulase-negative staphylococci appears to be chromosomal mediated.
6. Slime positive and negative colonies can not be differentiated by selectively staining with alcian blue and congo red.
7. Slime appears to be a poor immunogen in the rabbit model.

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APPENDICES

Appendix A

Buffers

Cell Lysis Buffers

Tris-Sucrose Buffer

1 M Tris-HCl, pH 8.0	5.0 ml
0.5 M EDTA, pH 8.0	1.0 ml
Sucrose	125 g
H_2O	494 ml

Triton Lytic Mix

10% Triton X-100 in 10 mM Tris-HCL, pH 8.0	1.0 ml
0.25 M EDTA, pH 8.0	25.0 ml
1 M Tris-HCl, pH 8.0	5.0 ml
H_2O	69.0 ml

DNA Suspension Buffers

Tris-EDTA-Saline Buffer (TES)

0.03 M Tris-HCl, pH 8.0

0.05 M EDTA, pH 8.0

0.005 M NaCl

Agarose Gel Electrophoresis Buffers

Tris-Borate Buffer

Boric Acid	22.0 g
Tris Base	43.2 g
EDTA (disodium)	3.72 g
H_2O	1000 ml

Appendix B

Reagents

Agarose Gel Electrophoresis Tracking Dye

Bromophenol Blue	0.07 g
SDS	7.0 g
Glycerol	33.0 ml
H_2O	67.0 ml