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An Extracellular Protein Produced by *Staphylococcus Hominis* Strain αM with Antimicrobial Activity Against *Mycobacterium* SPP

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AN EXTRACELLULAR PROTEIN PRODUCED BY *STAPHYLOCOCCUS HOMINIS* STRAIN αM WITH ANTIMICROBIAL ACTIVITY AGAINST *MYCOBACTERIUM* SPP.

by

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ABSTRACT

AN EXTRACELLULAR PROTEIN PRODUCED BY *STAPHYLOCOCCUS HOMINIS* STRAIN αM WITH ANTIMICROBIAL ACTIVITY AGAINST *MYCOBACTERIUM* SPP.

Sandra May Jacobsen
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The inhibitory activity exhibited by *Staphylococcus hominis* strain αM toward *Mycobacterium* species was examined. These studies included determining the conditions that maximized inhibitory agent production, analyzing characteristics of the agent, determining the time of agent production, ascertaining plasmid possession and the role of plasmids in the production of this inhibitory agent, and ascertaining the antimicrobial activity of other strains of *S. hominis*. On solid and in liquid media, *S. hominis* strain αM exhibits a unique inhibitory activity toward *Mycobacterium* species that is not demonstrated by *Staphylococcus epidermidis* or other *S. hominis* strains. Maximum agent production and recovery was accomplished when *S. hominis* αM, inoculated in either Tryptic soy broth or Todd-Hewitt broth, was incubated for at least 18 hours at 37°C under aerobic (shaking) conditions. The agent was subjected to one-step 60% ammonium sulfate precipitation and polyethylene glycol concentration. The molecular weight of the agent was estimated by membrane filtration to be between 12000 and 30000 MW and was characterized as containing a heat-stable, acid-stable, and base-labile biologically active protein moiety. This agent displays a broad spectrum of inhibitory activity toward gram-positive bacteria but showed little effect on gram-negative organisms. Growth of *S. hominis* strain αM consists of a lag phase, with

production of the agent occurring during logarithmic phase. *S. hominis* strain αM potentially possesses up to seven plasmids when examined electrophoretically and curing with various agents did not cease the anti-Mycobacterial activity.

To my significant other, Chris, my children, Alexandra and Matthew
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CHAPTER I

INTRODUCTION

Bacterial Antagonism and Secondary Metabolites

From the primordial soup that covered the primitive earth, evolution spawned bacteria, a lifeform which has continued to survive and flourish to the present day. Bacterial survival is dependent upon the ability of these organisms to adapt to an ever-changing, harsh environment. Bacteria are constantly competing with other bacteria for nutrients, etc. and when such essentials become limited, bacterial antagonism, caused by the production of various inhibitory metabolic by-products, may decrease competition and thus enhance chances of survival.

Antagonism between bacteria, first reported by Pasteur and Joubert in 1877, has intrigued researchers because of the potential for disease prevention and/or therapy (24). Several early bacterial antagonism studies that dealt with disease prevention and therapy included bacteriotherapy for anthrax and the implantation of lactic acid-producing bacteria into the intestine to reduce bacterial putrefaction products thought to affect the aging process (45). However, none of these early studies clearly identified the bacterial by-product responsible for the antagonism.

Most bacteria produce secondary metabolites, naturally produced substances which are not essential for growth (106). While the primary functions of many of these metabolites have yet to be determined, some exhibit activities which are antagonistic towards a variety of organisms including animals, plants, and microorganisms.

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Examples of antimicrobial secondary metabolites include bacteriolytic enzymes such as lysostaphin and hemolysins, antibiotics such as bacitracin and vancomycin, as well as bacteriocins and bacteriocin-like products (45). Lysostaphin, a bacteriolytic enzyme produced by staphylococci (e.g., *Staphylococcus simulans*) weakens the cell walls of susceptible staphylococci, such as *Staphylococcus aureus*, by the splitting of glycylglycine bonds in the pentaglycine bridge of peptidoglycan (109). Hemolysins, produced by a variety of gram-negative and gram-positive bacteria, are protein toxins which form pores in some prokaryotic and eukaryotic biological membranes (14). The α-hemolysin, produced by *Escherichia coli*, causes cytolysis in erythrocytes by the binding, insertion, and subsequent formation of pores, three nm. in diameter, in the plasma membrane. Examples of hemolysins produced by gram-positive bacteria include Streptolysin O, a thiol-activated hemolysin produced by *Streptococcus pyogenes*, and α-toxin, a hemolysin produced by *Staphylococcus aureus*.

Besides lysostaphin and hemolysins, certain antibiotics, such as bacitracin and vancomycin, also affect the cell walls of susceptible organisms. In general, antibiotics are defined as a diverse group of chemical substances produced by microorganisms or similar substances produced wholly or partially by chemical synthesis, which in low concentrations, interferes with the growth of other microorganisms (111). Bacitracin, a thiazoline ring-containing polypeptide antibiotic produced by strains of *Bacillus subtilis*, is bactericidal for many gram-positive bacteria and pathogenic *Neisseria* (111). Similarly, vancomycin, a narrow-spectrum, bactericidal, cell wall inhibiting antibiotic produced by *Streptomyces orientalis*, is active against various gram-positive organisms (111). The biosynthesis of antibiotics such as vancomycin and bacitracin occurs via

multienzyme complexes; in contrast, most bacteriocins and bacteriocin-like molecules are ribosomally synthesized as a simple inactive peptide that is posttranslationally modified and subsequently cleaved from a leader sequence to yield the active inhibitor.

Bacteriocins and bacteriocin-like molecules are a diverse group of secondary metabolites that can be generally characterized by the following: presence of a biologically active protein moiety, lethal biosynthesis, predominantly narrow spectrum of activity, attachment to specific receptors on the surface of bacteriocin-sensitive cells, and plasmid-borne genetic determinants for production and host cell immunity (45). Due to the numerous variations that exist amongst the bacteriocins, a more precise classification system has yet to be developed.

Bacteriocins of Gram-negative Bacteria

Classical bacteriocin definitions and characteristics were based on the first extensively studied group of bacteriocins, the colicins, which are produced by *Escherichia coli* (Table 1) and other closely related members of the family *Enterobacteriaceae*. In general, the bacteriocins of gram-negative bacteria are large molecular weight (29- to 90-kDa), heat-labile proteins with functional domains which specify attachment (central domain), translocation (amino terminal domain), and killing potential (carboxyl terminal domain) (45, 79). Colicins display a narrow spectrum of inhibitory activity, although some are toxic to eukaryotic cells. Examples of the bactericidal action of colicins include ion channel formation in energized cytoplasmic membranes which leads to diminished proton motive force, nuclease activity once they have entered susceptible cells, inhibition of protein synthesis by the inactivation of

TABLE 1. Colicins of *Escherichia coli*

Colicin	Mode of action	MW	Reference (s)
E1	Membrane depolarization	57279	17
A	Membrane depolarization	62989	66
E2	Nonspecific DNA endonuclease	61561	19
E3	Ribosome inactivation	60000	47
M	Peptidoglycan, O-antigen synthesis inhibition	29453	33

ribosomes, and inhibition of peptidoglycan and O-antigen synthesis (Table 1).

The structural gene for a colicin, as well as the protein responsible for producer cell immunity, and the lysis protein responsible for colicin release into the environment, are organized as operons located on plasmids (33). Immunity to colicin activity has been observed for the colicins with nuclease activity and the pore-forming colicins. Inactivation of colicins with nuclease activity occurs when the active peptide complexes with immunity proteins (47, 113). This is also observed for colicin M which binds to the immunity protein, Cmi, thereby, neutralizing the inhibitory action (33). The inactivation of the pore-forming colicins, such as E1, is known to be independent of complexing with an intrinsic inner membrane immunity protein (30). However, the manner in which this colicin and the immunity protein interact has yet to be determined. Once bacteriocin production is complete, colicin-producing bacteria must synthesize lysis proteins to allow for colicin release into the extracellular environment; this is the case because the colicin lacks a leader sequence. Lysis proteins destabilize the cell envelope through the activation of the detergent resistant phospholipase A which causes the cells to become leaky and thus release the intracellular build up of colicin (33). Thus, the production of colicins coincides with the producer cell death and therefore, the production of colicins can be induced by activators of the producer cells SOS response (33).

Once released into the extracellular environment, colicins interact with the membranes of susceptible organisms. Colicins adsorb to specific outer membrane receptors the primary role of which is the uptake of growth factors. However, a majority of these bound proteins are degraded before the inhibitory activity has begun (63). Only those colicins that bind receptors near Bayer sites, where the cytoplasmic membrane and

the outer membrane are in close juxtaposition, insert into the cytoplasmic membrane (63). Once inserted, the active colicin can begin its inhibitory activity by either remaining in the cytoplasmic membrane to form ion channels or by entering the cells and initiating its nuclease activity.

Bacteriocins of Gram-positive Bacteria

In depth studies investigating the bacteriocins of gram-positive bacteria began after the extensive studies were conducted on the colicins. According to Florey *et al.* (24), perhaps the first gram-positive bacteriocin-like agent was described by Babes in 1885; he observed antagonism between different staphylococcal species grown on solid media. These experiments on bacterial antagonism eventually led to the utilization of staphylococcal nasal and throat sprays for the treatment of diphtheria (45). In 1946, Fredericq reported that certain strains of staphylococci produced substances that inhibited the growth of other staphylococci and some other gram-positive bacteria, but not gram-negative bacteria. These agents are referred to as the staphylococcins (27). Following the discoveries of the staphylococcins and other antimicrobial substances produced by noncoliform bacteria, Jacobs *et al.*, in 1953, coined the general expression bacteriocin in order to group together the inhibitory agents produced by both gram-positive and gram-negative bacteria (46).

Classification of bacteriocins of gram-positive bacteria. As more inhibitory agents produced by gram-positive bacteria were reported, the need for a complete classification scheme for the bacteriocins of gram-positive bacteria increased. With the chemical diversity that exist amongst the bacteriocins of gram-positive bacteria, these

molecules can be grouped in different ways. One classification method, devised for the bacteriocins produced by lactic acid bacteria, defines four basic classes of bacteriocins (58). Class 1 consists of the lantibiotics. Class 2 includes small (<10-kDa) relatively heat-stable, non-lanthionine-containing membrane-active peptides, for example pediocin PA-1 and pediocin AcH, both of which are produced by strains of *Pediococcus acidilactici* (35, 68). Class 3 contains large (>30 k-Da), heat-labile proteins such as Helveticus J, produced by *Lactobacillus helviticus* 481 (49). Class 4 includes complex bacteriocins that contain essential lipids or carbohydrate moieties in addition to protein; examples include plantaricins S and T, bacteriocins produced by *Lactobacillus plantarum* LPCO10 which contain carbohydrate moieties (48).

Another method of classifying bacteriocins of gram-positive bacteria is based on the cysteine residue content in the mature active protein (45). The lantibiotics possess the cysteine-containing modified amino acid residues, lanthionine and β-methyllanthionine. These amino acids are formed by the electrophilic addition of cysteine with the dehydro forms of serine and threonine, dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively. Bacteriocins that lack the modified amino acids, lanthionine and β-methyllanthionine, but possess pairs of cysteines that form disulfide bridges are named cystibiotics. Examples of cystibiotics include the broad spectrum bacteriocins, pediocin PA-1 and pediocin AcH (35, 68). Thiolbiotics are those bacteriocins that contain a single cysteine residue. An example of this group includes lactococcin B, a narrow spectrum bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* 9B4 (102, 103). Lastly, there are bacteriocins that possess no cysteine residues such as lactococcin A, a narrow spectrum bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* LMG 2130 (38).

Lantibiotics. Production of lantibiotics has been observed in *Bacillus*, *Streptococcus*, *Staphylococcus*, *Lactococcus*, and *Streptomyces*. Lantibiotics can be further categorized based on their secondary structures (Table 2): those structures that resemble nisin (type A), and those that resemble duramycin (type B) (51). The type A lantibiotics are linear, cationic protein which are composed of up to 44 residues. The type B lantibiotics are small, globular proteins that can consist of as little as 19 residues. Unlike the nisin-like lantibiotics, the duramycin-like lantibiotics are less cationic or neutral.

General characteristics of bacteriocins of gram-positive bacteria and comparisons to bacteriocins of gram-negative bacteria. As previously mentioned, bacteriocins are generally characterized by certain criteria. For example, all bacteriocins, produced by gram-positive and gram-negative organisms, contain a biologically active protein moiety. However, variations in the protein structures of the bacteriocins of gram-positive bacteria exist; lactacin F, a narrow spectrum bacteriocin produced by *Lactobacillus johnsonii* VPI 11088 (Laf^+), is comprised of different proteins while plantaricins S and T contain carbohydrate moieties (2, 48). Apart from the presence of a biologically active protein moiety, differences exist between the bacteriocins of gram-negative and gram-positive organisms. Such differences include protein size, location of the genetic determinants of bacteriocin production and of host immunity, maturation process of the bacteriocins, producer strain immunity, spectrum of activity, host-bacteriocin interaction, modes of action, and time of production (45).

Protein sizes of mature bacteriocins vary greatly between gram-positive and gram-negative bacteria. As indicated earlier, the gram-negative colicins are generally higher

TABLE 2. Types of lantibiotics

Name	Mass (Da)	No. amino acids[a]	Producer organism	Gene[b]	Reference (s)
Type A: Linear					
Nisin A	3353	34	*Lactococcus lactis* 11454	Both	31
Nisin Z	3330	34	*Lactococcus lactis*		69
Subtilin	3317	32	*Bacillus subtilis* strain ATCC 6633	Chromosome	6
Epidermin	2164	22	*Staphylococcus epidermidis* Tü3298	Plasmid	92
1V,6L epidermin	2151	22	*Staphylococcus epidermidis* spp.		42
Gallidermin	2164	22	*Staphylococcus gallinarium* Tü3928	Chromosome	91
Pep5	3488	34	*Staphylococcus epidermidis* strain 5	Plasmid	54
Epilancin K7	3032	31	*Staphylococcus epidermidis* K7	Chromosome	105
SA-FF22	2795	26	*Streptococcus pyogenes* FF22	Chromosome	41, 101
Lacticin 481	2901	28	*Lactococcus lactis* subsp. *lactis*	Plasmid	78, 82
SalivaricinA	2315	22	*Streptococcus salivarius* 20P3	Chromosome	83
Mutacin	3245	25	*Streptococcus mutans* T8	Chromosome	75
Lactocin S	3764	37	*Lactobacillus sake* strain α45	Plasmid	67
Cytolysin L1	4164	44	*Enterococcus faecalis*	Plasmid	28
Cytolysin L2	2631	27	*Enterococcus faecalis*	Plasmid	28
Carnocin UI 49	4635	37	*Carnobacterium piscicola*		96
Type B: Globular					
Cinnamycin	2041	19	*Streptoverticillium* & *Streptomyces* spp.	Chromosome	53, 56
Duramycin A	2012	19	*Streptoverticillium* & *Streptomyces* spp.		25, 26
Duramycin B	1951	19	*Streptoverticillium* spp.		25, 26
Duramycin C	2008	19	*Streptomyces griseoluteus*		25, 26
Ancovenin	1959	19	*Streptomyces* spp.		107
Mersacidin	1825	20	*Bacillus subtilis* HIL Y-85, 54728	Chromosome	9
Actagardine	1890	19	*Actinoplanes* ATCC 31048 & 31049		57, 114

[a] The number of amino acid residues contained in the propeptide.
[b] The location of the genes responsible for lantibiotic production and immunity if known.

molecular weight than the bacteriocins of gram-positive bacteria. Even though some gram-positive bacteria produce high molecular weight, heat-labile bacteriocin-like proteins similar to the colicins, such as the 37 kDa Helveticus J (49), many bacteriocins from gram-positive bacteria are low molecular weight, heat-stable, cationic proteins. Examples of these low molecular weight proteins include the lantibiotics, nisin and SA-FF22 and the non-lantibiotics, pediocin AcH and pediocin PA-1 (41, 45).

As with all proteins, the lanthionine and non-lanthionine containing bacteriocins, as well as the proteins required for their production and immunity, are genetically encoded. The genes for bacteriocin structure, transcriptional regulation, posttranslational modification processing, translocation to the cell exterior, and producer strain immunity are arranged in multigene operons or regulons (45). In contrast to the plasmid-encoded production and immunity genes of the bacteriocins of gram-negative bacteria, the operons for the bacteriocins of gram-positive bacteria can be located either on plasmids or on the chromosome. Examples of bacteriocins of gram-positive bacteria whose operons are located on plasmids include the lantibiotics, Pep5 and epidermin, and non-lantibiotics, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A, and leucocin A-UAL187, produced by *Leuconostoc gelidum* UAL 187 (34, 37). The genes for production and immunity of bacteriocins of gram-positive bacteria have also been located on the chromosome, as is the case for the structural genes of the lantibiotic mersacidin and the non-lantibiotic bacteriocin sakacin 674, produced by *Lactobacillus sake* Lb674 (36, 102). However, the genes responsible for the production of the lantibiotic nisin have been found to be located on a transposon, Tn5301/Tn5276 (15, 29, 39, 79), hence may be present in either a plasmid or a chromosome.

After transcription of the genes that encode the bacteriocin peptide, subsequent translational process produces a mature active bacteriocin. This processing differs between the bacteriocins of gram-positive and gram-negative bacteria. The protein domains of colicins and many antimicrobial substances are produced through the formation of multienzyme complexes. In comparison, lantibiotics and non-lanthionine containing bacteriocins are ribosomally synthesized as an inactive prepeptide that is subsequently cleaved from a leader sequence into an active propeptide (12, 45). The leader sequence, located at the amino terminus of the prepeptide, is responsible for keeping the propeptide configuration stabilized and inactive before secretion into the extracellular environment. Prior to leader sequence cleavage, the propeptide, located at the carboxyl terminus of the prepeptide, may be posttranslationally modified, such as occurs with the lantibiotics (45). The formation of the unique amino acids, lanthionine and β-methyllanthionine, contributes to the configuration stability of the lantibiotic propeptide.

Another example of posttranslational modification processing is the maturation process of the non-lanthionine-containing bacteriocin, pediocin AcH (16). Prepediocin posttranslational modification occurs after its translocation through the cytoplasmic membrane. These modifications include formation of two disulfide bridges, activation of the prepediocin, and subsequently cleavage of the propeptide from the leader sequence by an endopeptidase, just prior to release into the extracellular environment.

During the production of a bacteriocin, producer strains, both gram-positive and gram-negative, must exhibit immunity or protection from their own inhibitory agent in order to survive the lethal effect of the active inhibitory agent. The gram-positive

producer strains and some of the gram-negative producer strains, such as colicin classes E2 and E3, have immunity against these active peptides by means of membrane associated molecules (45, 79). Both the production and immunity to the lantibiotic Pep5 have been correlated with the possession of plasmid pED503 (57). Analysis of the PepI, the gene product of *pepI*, suggested this peptide interacts loosely with the cytoplasmic membrane and serves to protect the host cell from the effects of the bacteriocin (79). Examples of immunity towards the non-lanthionine containing bacteriocins include a sakacin A immunity protein which is homologous to transmembrane protein kinases involved in various adaptive response systems in bacteria (5) while immunity for lactococcin A, a non-lanthionine containing bacteriocin, is based on blocking the target receptors or by inactivating the bacteriocin (104).

Those bacteria which are killed on exposure to the mature bacteriocin are said to be susceptible. As mentioned previously, the bactericidal action of the colicins effects only a small or narrow spectrum of organisms. In contrast, most bacteriocins of gram-positive bacteria display a broad spectrum of inhibitory activity. In most cases, this spectrum includes organisms within the same genera as well as other genera. The lantibiotic nisin, for example, inhibits many strains of gram-positive bacteria, such as staphylococci, streptococci, bacilli, clostridia, and mycobacteria (41).

After a bacteriocin is released into the environment, the first stage of interaction between the bacteriocin and its susceptible host is the physical contact. Colicins adsorb to receptors located on the outer membrane of susceptible hosts. In contrast to this, cationic bacteriocins of gram-positive bacteria appear to interact with the anionic membrane constituents, such as teichoic acid and lipoteichoic acid, of the susceptible host

(8, 11). Receptor binding to gain access to susceptible cytoplasmic membranes appears less necessary for the bacteriocins of gram-positive bacteria since gram-positive bacteria lack the outer membranes of gram-negative bacteria and their cell walls allow relatively large molecules to pass (45, 89).

After the initial interaction with the susceptible host, there appears to be two modes of bactericidal action exhibited by bacteriocins of gram-positive bacteria: barrel stave pore formation and induction of autolysis. The primary bactericidal activity of bacteriocins of gram-positive organisms is the formation of barrel stave pores that cause the influx of small molecules and the efflux of ions, amino acids, etc. from the cytoplasm (60, 103). This leads to the dissipation of the proton motive force and eventually to cell death. Differences exist between non-lantibiotics and lantibiotics in terms of the requirement of a membrane potential for adequate bacteriocin function.

Non-lantibiotics, such as lactococcin A and lactacin F, cause the cytoplasmic membrane of susceptible cells to lose its selective permeability barrier in a voltage independent manner. The pores generated by these bacteriocins appears to destabilize membrane function, such as by effecting the energy transduction systems of the susceptible cells rather than disrupting the structural integrity of the membrane. However, pore formation by lantibiotics is voltage dependent; in other words, energy provided by the membrane potential of an energy transducing membrane is required for insertion. When present in micromolar amounts, the lantibiotics, Pep5, subtilin, and epidermin require the membrane threshold level of energization, an energy amount of -80 to -100 mV, to form pores. However, the lantibiotic nisin only requires -50 mV (85). Therefore, eukaryotic cells, such as lung fibroblasts, and mycoplasma cells, which

membranes potentials range from -10 to -50 mV, are resistant to these lantibiotics (60). However, in millimolar amounts, these lantibiotics produce physical destabilization in all the membranes that were examined. Voltage dependency for lantibiotic insertion is demonstrated by the efflux of preaccumulated radiolabelled amino acids from artificially energized cytoplasmic membrane vesicles of *Bacillus subtilis* treated with the lantibiotics, nisin, Pep5, subtilin, and SA-FF22 (44, 61, 84, 88, 93). The lantibiotics, nisin and Pep5, also inhibited the biosynthesis of DNA, RNA, proteins, and polysaccharides (88).

Besides the formation of barrel stave pores, some lantibiotics have been shown to induce autolysis in susceptible hosts. For example, the lantibiotics, Pep5 and nisin, have been associated with the induction of autolytic enzymes in *Staphylococcus simulans* 22 (10).

Although the functional reason for bacteriocin production in producer strains remains a mystery, experiments determining their time of production continues to be examined. Prior to bacteriocin production, some Staphylococci capable of producing staphylococcins demonstrate the virtual absence of a lag phase during growth, while in strains devoid of a bacteriocinogenic factor, the lag phase is prolonged (43); This abbreviated lag phase is observed in *S. aureus* strain IYS2, a staphylococcus isolated from human saliva (72). Bacteriocin production has been observed at various phases during growth. For example, the staphylococcin, IYS2, is present in detectable amounts throughout logarithmic phase, while the lantibiotic, lactocin S, is produced only during late logarithmic phase (67, 72). However, other bacteriocins of gram-positive bacteria, such as Bac1829, a bacteriocin produced by *S. aureus* KSI1829 and the lantibiotic mersacidin, as well as some colicins, are produced in early stationary phase (18, 72).

Several bacteriocins of gram-positive bacteria, including IYS2 and Bac1829, appear to accumulate by the onset of stationary-phase growth and these levels of bacteriocin are maintained after 24 hours (21, 72).

Staphylococcus

Staphylococcus, a genus in the family *Micrococcaceae*, consists of irregularly clustered gram-positive facultative cocci. Various characteristics distinguish these organisms from other gram positive cocci. These bacteria are resistant to erythromycin (0.04 μg per ml), salt tolerant, catalase positive, oxidase negative, and non-motile. Staphylococci are susceptible to lysostaphin (200 μg per ml), while other gram-positive organisms such as Enterococci, Streptococci, Planococci, Stomatococci, Micrococci, and Aerococci are not susceptible. Of the more than thirty species of Staphylococci, five are clinically significant: *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*, and *Staphylococcus epidermidis* (59). As with other gram-positive cocci, some of these Staphylococci produce bacteriocins and other secondary metabolites.

S. aureus, a facultative anaerobe, is a common human parasite known to cause a variety of diseases including toxic shock syndrome, staphylococcal food poisoning, and pneumonia. These bacteria grow on various nutrient agars at temperatures ranging from 6.5 to 46°C, with an optimum between 30 to 37°C (112). On agar plates, most *S. aureus* appear as yellowish, smooth, opaque, round colonies, 1 to 4 mm in diameter (112). Several characteristics distinguish *S. aureus* from other staphylococci. One feature is a unique protein found in the cell wall of these organisms called Protein A, a group antigen

specific for most strains of *S. aureus* (112). Secondly, these bacteria produce coagulase, an extracellular enzyme that clots plasma, which distinguishes this species from other less pathogenic staphylococci (112).

S. aureus also produce a variety of extracellular enzymes, toxins and antimicrobial agents. Some examples of the extracellular enzymes produced by *S. aureus* include lipases, hyaluronidase, and nucleases. Lipase and hyaluronidase production facilitate the invasion and spread of these organisms in healthy subcutaneous tissues (112). The heat resistant nucleases produced by *S. aureus* cleave either DNA or RNA and act as either an endonuclease or an exonuclease.

S. aureus produces various toxins including hemolytic toxins, such as α toxin, and others that are able to cause shock, for example TSST-1. These bacteria also produce a variety of inhibitory agents, such as the staphylococcins. Staphylococcins produced by *S. aureus*, like those of other staphylococcal strains, are bacteriocins that inhibit a wide variety of gram-positive bacteria (87).

Some of the known inhibitory agents and staphylococcins produced by *S. aureus* are shown in Table 3. These inhibitory agents were isolated using a number of different techniques that include ammonium sulfate precipitation (21, 72), chromatographic fractionation on XAD-2 (94), gel filtration (21, 52), reversed-phase HPLC (21, 94), and ion-exchange chromatography (52, 72).

Like the coagulase-positive *S. aureus*, several coagulase-negative staphylococci are implicated in various human infections. *S. saprophyticus*, found on human skin and urethral regions, causes urinary tract infections in young, sexually active women (59). *S. haemolyticus* has been identified as a cause of urinary tract infections, peritonitis and

native valve endocarditis, while *S. lugdunensis* is known to cause brain abscesses and prosthetic valve endocarditis (59). *S. epidermidis*, perhaps the most common organism found on human skin and mucus membranes, causes many opportunistic hospital-acquired bacteremias (59). These organisms produce white colonies, require biotin to grow (112) and produce various inhibitory agents (Table 4).

As with the inhibitory agents of *S. aureus*, the inhibitory agents produced by strains of *S. epidermidis* have been isolated in various ways. These techniques include chromatography methods such as gel filtration (87, 110), cation exchange chromatography (86, 108), high-performance liquid chromatography on reversed-phase C18 column (86, 108) and adsorption to XAD 2 (86, 87, 108).

Two of the most studied lantibiotics, Pep5 and epidermin, are produced by *S. epidermidis* and studies on these two particular inhibitory agents have to lead to a better understanding of the genetics and mode of action of this type of inhibitory agent. Pep5, a 34 residue, heat-stable, bactericidal protein, is produced by *S. epidermidis* strain 5 (88). The genes responsible for the production, immunity, etc. involved with Pep5, are encoded in an operon located on a 18.6 kbp plasmid (pED503) and are arranged as followed: *pepTIAPBC* (22, 54, 65, 81). The structural and the immunity gene for Pep5 are *pepA* and *pepI*, respectively (54). In order for the producer strain to exhibit immunity, both *pepA* and *pepI* must be present (80). The two genes, *pepB* and *pepC*, encode the enzymes responsible for posttranslational modification of the propeptide (65). The gene, *pepT*, encodes for a translocator from the ABC transporter family that transports the propeptide to the extracellular environment while *pepP*, encodes for a serine protease that may function in cleaving of the leader sequence from the Pep5 prepeptide (65).

TABLE 3. Inhibitory agents of *S. aureus*

Name	Producer strain[a]	MW	Heat[b]	Spectrum[c]	Reference (s)
Au-26	Strain 26	2700	Stable	Lactobacilli	49
Bac1829	Strain KSI1829	6418	Stable	Broad	21
D91	Strain D91	76000	Labile	Broad	52
IYS2	Strain IYS2	5000	Stable	Oral bacteria	72

[a] Strains of *Staphylococcus aureus*.
[b] Cultures treated with heat to 100°C.
[c] Spectrum includes organisms inhibited by the agent.

TABLE 4. Inhibitory agents of *S. epidermidis*

Name	Producer strain[a]	MW	Heat[b]	Spectrum[c]	Reference (s)
115	Strain 115	6500	Stable	Turkey *S. aureus*	110
Epidermin/1580[d]	Strains Tü3298 and 1580	2164	Stable	Gram-positive	1, 86, 92, 98, 108
1V,6L epidermin	Strains BN-V1 and BN-V301	2151			42
Epilancin K7	Strain K7	3032			105
Pep5	Strain 5	3488	Stable	Broad	88

[a] Strains of *Staphylococcus epidermidis*.
[b] Cultures treated with heat to 100°C.
[c] Spectrum includes organisms inhibited by the agent if known.
[d] These agents were found to be similar.

The primary mode of action which Pep5 exhibits toward staphylococci and micrococci is the rapid abolishment of the PMF that inhibited the synthesis of DNA, RNA, protein and polysaccharide (84, 88). Pep5 reversibly binds electrostatically to anionic sites on the cell wall, such as teichoic acid and lipotechoic acid (84, 88). Pep5 has been shown to exhibit a secondary mode of action by inducing glucose-stimulated autolysis in *S. simulans* 22 (10).

Likewise, epidermin, a 22 amino acid lanthionine-containing peptide, is produced by *S. epidermidis* Tü3298. Epidermin inhibits pathogenic gram-positive bacteria such as *Propionibacterium acnes*, staphylococci and streptococci (92). This peptide contains a highly hydrophilic amino terminus and a highly lipophilic carboxy terminus.

The genes involved with the production and immunity of epidermin are encoded on a 54 Kb plasmid called pTü32 (92). This plasmid contains nine genes arranged in the following operons: *epiFEG*, *epiT*, *epiABCD*, and *epiQP* (62, 77, 90, 92). The gene, *epiA*, encoding the structural protein of epidermin, is cotranscribed with *epiB*, *epiC*, and *epiD*, the genes encoding for the proteins involved with posttranslational modification. The genes implicated in the regulation and maturation of epidermin synthesis, *epiP* and *epiQ*, are located downstream from but in the opposite orientation to *epiABCD* (62, 90, 92). Epi P has homology to several serine proteases, and may function in cleaving of the leader sequence from the epidermin prepeptide while EpiQ appears to be responsible for the activation or positive regulation of the epidermin operon (77). Three genes have been found to be involved in immunity of the producer strain: *epiF*, *epiE* and *epiG*. The gene, *epiT*, encodes for a protein, EpiT, that structurally resemble ABC transporter proteins such as HlyB (23), the peptide responsible for the extracellular secretion of α-hemolysin

of *E. coli*, and this protein may have a role in transporting the epidermin peptide to the extracellular environment.

Natural variants of epidermin have been discovered. One, a 2151 Da bacteriocin isolated from two *S. epidermidis* strains, BN-V1 and BN-V301, is named (Val1, Leu6)-epidermin (42). Another, isolated from *Staphylococcus gallinarium* (F16/P57) Tü3928, is called gallidermin or 6L-epidermin (55). Gallidermin, a chromosomal-encoded type-A lantibiotic, varies from epidermin by a Leu/Ile exchange in position 6 (55).

Staphylococcus hominis

Staphylococcus hominis is a catalase-positive, oxidase-negative, salt tolerant, lysostaphin-susceptible, low erythromycin- resistant, non-motile facultative anaerobe. This organism grows within 24 hours of inoculation, forms 2.5 to 6 mm dia. smooth opaque colonies and adheres loosely on agar (59). *S. hominis* can be further distinguished from other gram-positive cocci and staphylococcal species by the characteristics listed in Table 5.

This organism is found on humans and primates especially in larger populations on the skin where apocrine glands are numerous, such as axillary and pubic areas. *S. hominis* has been implicated in septicaemia in cancer patients (13).

Interest in Bacteriocins of Gram-positive Bacteria

The interest in lantibiotics and other bacteriocins of gram-positive bacteria grows because of their potential therapeutic properties and their potential use in food preservation. Many lactic acid bacteria are food grade gram-positive bacteria involved in

TABLE 5. A comparison of various characteristics of *S. hominis*, other staphylococcal species and other gram-positive cocci

Organism	Catalase	Lysostaphin[a]	Ery.[b]	Large colony	Anaerobe	Aerobe	Hemolysin	Nuclease[c]	BG[d]	AP[e]
S. hominis	+	+	+	-	-	+	-	-	-	-
S. aureus	+	+	+	+	+	+	+	+	+	+
S. epidermidis	+	+	+	-	+	+	sd	-	sd	+
S. saprophyticus	+	+	+	+	d	+	-	-	s	-
S. haemolyticus	+	+	+	+	d	+	d	-	s	-
Enterococcus	+	+	+							
Streptococcus	-	-	-							
Aerococcus	-	-	+							
Planococcus	-	-	+							
Micrococcus	-	-	-							
Stromatococcus	-	-	+							

[a] Susceptible to 200 µg/ml lysostaphin.
[b] Resistant to 0.04 µg/ml erythromycin.
[c] Heat stable nuclease.
[d] β-glucosidase.
[e] Alkaline phosphatase.
+ Positive response in ≥ 90% of the colonies.
- Negative response in ≥ 90% of the colonies.
d Delayed positive response in ≥ 90% of the colonies.
s 11-89% of the colonies are positive.
sd Delayed positive response of 11-89% of the colonies.

food fermentation production processes. These organisms have been observed to inhibit the growth of various food spoilage organisms. This is the case with dairy products inoculated with nisin-producing *Lactococcus lactis* in dairy fermentations (45). Since nisin has exhibited a bacteriostatic mechanism similar to nitrate, it may be utilized in foods as a substitute preserver replacing the cancer-causing nitrate (15).

Several bacteriocins of gram-positive bacteria, including various lantibiotics, have potential therapeutic significance as a primary or alternative method of treatment for numerous ailments. The lantibiotics, epidermin and gallidermin have been utilized as a treatment for acne because of their ability to inhibit staphylococci and *P. acnes* (59). Ancovenin, a type B lantibiotic, has been demonstrated to inhibit angiotensin-converting enzyme, thus, a possible treatment for high blood pressure (107). Cinnamycin (also known as lanthiopeptin or Ro 09-0198), a type B lantibiotic that inhibits phospholipase A_2, shows antiviral activity towards the Herpes simplex virus 1 (64, 73, 95).

There are many aspects of bacteriocins of gram-positive organisms that make these inhibitory agents favorable for use as therapeutic agents. First, most bacteriocins of gram-positive bacteria display a broad spectrum of inhibition, thus could be used against a variety of potentially pathogenic organisms. Secondly, most known bacteriocins of gram-positive bacteria are ribosomally synthesized as a single peptide. In contrast, common antibiotics like the β-lactam antibiotics, (e.g., penicillin), and peptide antibiotics, (e.g., gramicidin and valinomycin), are produced by multienzyme complexes therefore complicating the manufacturing of laboratory produced analogues for alternative drug therapies (32, 45).

Since research on several of the bacteriocins of gram-positive bacteria (epidermin,

gallidermin, ancovenin, and cinnamycin) has shown potential therapeutic significance, further bacteriocinogenic agents may also prove beneficial. Research on the effectiveness of various inhibitory agents, such as the bacteriocins of gram-positive bacteria, must continue because of the need for alternative drug treatments due to the development of new antibiotic resistant strains. *Mycobacterium tuberculosis*, the organism responsible for the disease tuberculosis, has become resistant to one or several of the first-line antibiotics (isoniazid, rifampin, streptomycin, ethambutol) utilized to treat this disease because of improper antibiotic usage and patients not completing the prescribed drug regimen; such non-compliance with treatment has allowed the organisms time to evolve and develop resistance (74, 76). Alternative antibiotics for this curable disease are expensive and the potential overusage of these antibiotics could result in the development of new strains resistant to all current drug treatments. This situation would cause a potentially serious dilemma, for even though this disease is curable when detected early and treated, tuberculosis is fatal when not treated. Therefore, the discovery and development of agents that inhibit *M. tuberculosis* could provide a solution for this potential problem.

This project focuses on one particular strain of *S. hominis*, *S. hominis* strain αM, which has antimicrobial activity against certain *Mycobacterium* species, in particular *M. phlei* and *M. smegmatis*. To characterize the antimicrobial agent responsible for the anti-Mycobacterial activity, the following properties were investigated: conditions that influenced inhibitory agent production, physical and chemical characteristics, determination of antimicrobial activity of other strains of *S. hominis*, time of production of the agent, plasmid possession of *S. hominis* strain αM, and association between the

presence of plasmids and production and immunity of the inhibitory agent.

Various conditions were examined to determine those that influenced inhibitory agent production. First, screening of the potential producing organism for bacterial antagonism on various solid media by the deferred antagonism method and in various liquid media by the well diffusion method was used to ascertain the ability to support inhibitor production. The optimal growing condition for maximum anti-Mycobacterial agent production was ascertained by incubating *S. hominis* strain αM under various aerobic, temperatures and aeration conditions. Concentration of the inhibitory agent responsible for the bacterial antagonism was attempted through utilization of certain techniques used for non-lanthionine type bacteriocins (ammonium sulfate precipitation and polyethylene glycol concentration).

Various physical and chemical characteristics of the inhibitory agent, including properties such as molecular weight approximation, susceptibility to heat, certain enzymes, and pH, and the spectrum of inhibitory activity were investigated.

The anti-Mycobacterial activity of *S. hominis* strain αM was compared to other strains of *S. hominis* by examining the inhibitory agent production on a solid media and in a liquid media. The time of inhibitory agent production was ascertained by analyzing the growth of *S. hominis* strain αM with inhibitory agent production in a liquid media.

Finally, *S. hominis* strain αM, as well as other *S. hominis* strains, were examined for the presence of plasmid. Afterwards, *S. hominis* strain αM was treated with the curing agents, mitomycin C, rifampicin, and ethidium bromide, to determine any association between the presence of plasmids and production and immunity of the inhibitory agent.

CHAPTER II

MATERIALS AND METHODS

Materials

Bacteria and culture maintenance. The inhibitor producing strain, *S. hominis* strain αM, was obtained as a natural isolate and identified a *S. hominis* by microscan analyses. *S. hominis* strains ATCC 27845, ATCC 27847, E54A-2, and E36B-4 were obtained from Dr. W. E. Kloos, Department of Genetics, North Carolina State University. Inhibitor production assay indicators, *Mycobacterium phlei* and *Mycobacterium smegmatis*, were obtained from the culture collection of the Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City. *S. epidermidis* 5, a staphylococci utilized for comparison, and the indicator strain *Micrococcus luteus* I1, were obtained from Dr. J. R. Tagg, University of Otago, Dunedin, New Zealand. The yeast and bacterial indicator strains utilized for the spectrum studies were from the culture collection of the Biological Sciences Department, Old Dominion University, Norfolk, VA and included *Staphylococcus aureus* ATCC 25823, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 4698, *Enterococcus faecalis* ATCC 19433, *Aerococcus viridans, Streptococcus bovis, Streptococcus pneumoniae. Streptococcus salivarius, Streptococcus mutans,* Group A Streptococcus ATCC 19615, Group B Streptococcus, Group C Streptococcus, Group F Streptococcus, Group G Streptococcus, *Bacillus subtilis* ATCC 6051, *Bacillus subtilis* ATCC 6054, *Bacillus cereus* ATCC 14579, *Bacillus stearothermophilus* ATCC 12980, *Corynebacterium kutscheri* ATCC 15677, *Corynebacterium xerosis* ATCC 373, *Corynebacterium xerosis* ATCC 83, *Mycobacterium phlei*, *Mycobacterium smegmatis*,

Erysipelothrix rhusiopathiae, *Enterobacter aerogenes* ATCC 18048, *Enterobacter cloacae* ATCC 23355, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 131, *Acinetobacter anitratus* ATCC 19606, *Citrobacter freundii* ATCC 8090, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14024, *Serratia marcescens* ATCC 13048, *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931, *Shigella flexneri* ATCC 12222, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas denitrificans*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Alcaligenes faecalis*, *Branhamella catarrhalis*, *Saccharomyces cerevisiae* and *Candida albicans*.

Cultures were grown on Todd-Hewitt agar plates and incubated at 35°C for 24 hours, except for the Mycobacteria cultures which were incubated for 48 hours prior to use. All cultures were maintained at 4°C for up to one month.

Media. Media utilized was purchased from Difco Laboratories, Detroit, MI and was prepared according to manufacturer's instructions. Media included the following : Todd-Hewitt broth (THB), Columbia Blood Agar Base (CBAB), Tryptic Soy (T-Soy), Brain Heart Infusion (BHI), and Blood Agar (BA). Supplements were added as required, either by presterilization or as a sterile addition following preparation.

The preparation of solid media was accomplished by the addition of 1.5% (w/v) Bacto-Agar (Difco) to the liquid broth prior to autoclaving. The agar was poured into 100 X 15 mm polystyrene petri dishes (Baxter Healthcare Corp., McGaw Park, IL); solidified plates were stored at 4° C. Liquid media, after autoclaving, was stored at room temperature (RT).

Chemicals. The following chemicals were obtained from Fisher Scientific, Fairlawn, NJ: sodium chloride (enzyme grade), potassium chloride (enzyme grade),

sodium phosphate hydrated, potassium dihydrogen phosphate (monobasic, crystal, enzyme grade), Tris base (molecular biology grade), EDTA disodium salt (electrophoresis grade), glucose, sodium hydroxide (molecular biology grade), potassium acetate (enzyme grade), isoamyl alcohol (biotechnology grade), chloroform (molecular biology grade), boric acid (electrophoresis grade), ammonium sulfate, polyethylene glycol (Carbowax® PEG 8000),

ethanol, hydrochloric acid, saturated phenol pH 6.6, agarose (electrophoresis grade), ethidium bromide (electrophoresis grade), and sucrose (ultracentrifugation grade).

The following enzymes and chemicals were obtained from Sigma Chemicals Co., St. Louis, MO: proteinase-K, α-chymotrypsinogen-A (Type II, bovine pancreas), protease p-6141 (Type IX, from *Bacillus polymyxa*), protease p-5647 (Type XV, from *Bacillus polymyxa*), lysostaphin (*Staphylococcus simulans*), RNAse, bromophenol blue, xylene cyanole FF, sodium dodecyl sulfate, erythromycin and rifampicin.

Other hydrolytic enzymes utilized were from by the following. Lysozyme (hen egg whites) was purchased from Calbiochem, San Diego, CA. Trypsin 1-250 (pig pancreas) was from US Biochemicals Corp., Cleveland, OH and lipase from Carolina Biological Supply Co., Cabisco Chemicals, Burlington, NC. Restriction enzymes and their reaction buffers were purchased from Promega, Madison, WI and Life Technologies, Gibco BRL, Gaitherburg, MD and utilized according to manufacturer's instruction.

Glycerol was from Boehringer Mannheim, Indianapolis, IN.

Bovine Serum Albumin, use as the standard for the 280 nm protein assay, was purchased from Pierce Biochemicals, Rockford, IL.

Dialysis membrane tubing with a molecular pore size of 12000 to 14000 MW was purchased from Spectral Por, Spectrum Co., Gardena, CA.

Mitomycin C was purchased from Acros Organic (Geel, Belgium).

Methods

Determination of the antimicrobial activity on solid media by the deferred antagonism method. Inhibitor activity was detected in solid media by the deferred antagonism method (97). This technique was used to examine the ability of various agars (TH, CBAB, T-Soy, BHI, and BA) to support inhibitor production and in determining the inhibitory spectrum of the antimicrobial agents. When examining inhibitor production on various agars, *S. epidermidis* 5, was utilized for comparison. A one-cm wide streak of the test strain was inoculated diametrically across the surface of various media using a sterile cotton swab seeded with an 18 hour, aerobically grown (37°C) THB + 1% yeast extract culture. The inoculated plates were incubated aerobically for 18 hours at 35°C after which the bacterial growth was removed by scraping the agar plate surface with a glass slide edge. The agar surface was sterilized by exposure to chloroform vapors for one hour then aired for 30 minutes to allow for escape of residual chloroform vapors. Indicator strains were streaked perpendicular to the original strip using a sterile swab seeded with an 18 hour aerobically (37°C) grown THB cultures. When *M. phlei* and *M. smegmatis* were utilized as the indicator strains, an additional 24 hours of growth was required prior to their use as an inoculation onto the agar plates. After incubation (aerobically for 48 hours at 37°C), the degree of inhibition was measured by the following scale: - represents no inhibition, +/- represents partial inhibition with single

colony growth within the area of the original strip, + represents weak inhibition, and ++ represents strong inhibition.

Determination and assaying of the antimicrobial activity in liquid media by the well diffusion method. Inhibitor production in liquid media was detected by a modified version of the well diffusion method (50). Various liquid media, such as THB + 1% yeast extract, T-Soy, BHI, and THB, were assayed for inhibitor production as follows. The producer strain *S. hominis* strain αM and *S. epidermidis* 5 were inoculated in the test liquid and incubated aerobically for 18 hours at 37°C. The culture was centrifuged (two minutes, RT, 14000 X *g*) and the supernatant retained. Six-mm. wells were punched into TH agar plates utilizing the unpulled end of a glass pasteur pipette. An aliquot of each test broth was pipetted into a well and allowed to air dry, for usually one hour, until the liquid was entirely absorbed. The agar plates were sterilized by exposure to chloroform vapors for one hour and then aired for 30 minutes prior to seeding the indicator lawn. The bacterial lawn was swabbed over the entire surface of the agar using an 18 hour (*M. luteus* I1 and *S. epidermidis* 5) or a 48 hour (*M. phlei* and *M. smegmatis*) culture. The plates were incubated aerobically at 35°C for 48 hours. The diameter of the zone of inhibition, indicated by a zone of no growth, was measured in mm.

The titre of the inhibitory agent was determined utilizing a series of two fold dilutions of the test solution. The highest dilution of the test solution to yield a detectable inhibition zone was expressed as containing one arbitrary unit of inhibitory activity (AU).

Optimal conditions for anti-Mycobacterial activity production. Various environmental factors were investigated to ascertain the conditions which influence the production of the anti-Mycobacterial activity in a liquid broth. An overnight culture of *S.*

hominis strain αM was diluted 20-fold in fresh THB and incubated for 24 hours under different conditions: candle jar at 32°C, aerobically at 32°C, aerobically at 55°C, anaerobically at 35°C, 37°C in a shaking water bath, and 37°C in a standing water bath. After overnight incubation, the cultures were centrifuged (two minutes, RT, 14000 X *g*) and assayed for inhibitory activity by the well diffusion method utilizing *M. smegmatis* as the indicator. The size of the zones of inhibition was measured in mm.

Concentration of the inhibitory agent from liquid media. Based on the preliminary results of the physical and chemical characteristics of the inhibitory agent, the isolation and purification procedures were approached as if the inhibitory agent possessed a protein moiety. Each step of the concentration process was examined to determine which procedures maximized recovery of the inhibitory agent. To obtain the crude extract, the inhibitor producing strain, *S. hominis* strain αM, was inoculated in 125 ml bottles containing 50 ml THB and was incubated for 24 hours at 37°C in a shaking water bath. The cell free supernatant, prepared by centrifugation (20 minutes, 4°C, 10000 X *g*), represents the crude extract. This extract was subjected to the following procedures (12).

To concentrate the inhibitory agent, the crude extract was first subjected to precipitation with ammonium sulfate. To ascertain the concentration of ammonium sulfate that precipitates the maximum amount of detectable inhibitory agent, a step-wise ammonium sulfate procedure (50% to 80% ammonium sulfate) was performed (12). Solid ammonium sulfate was added slowly to the crude extract to a concentration of 50% with gentle constant stirring (on ice, one hour). The precipitated proteins were collected by centrifugation (20 minutes, 4°C, 10000 X *g*) and the pellet resuspended in a minimal amount of deionized water. Ammonium sulfate was added to the remaining supernatant

in a step-wise manner (60% to 80%). Following each addition of ammonium sulfate, the precipitated proteins were collected by centrifugation (20 minutes, 4°C, 10000 X *g*) and the pellet resuspended in a minimal amount of deionized water.

The inhibitory agent was also precipitated by a one-step ammonium sulfate precipitation. The ammonium sulfate precipitation on the crude extract was performed essentially as described in the previous section, except ammonium sulfate to 80% was added in one step. Following resuspension of the pellet obtained by ammonium sulfate precipitation, dialysis against phosphate buffered saline (8 mg per ml sodium chloride, 0.2 mg per ml potassium chloride, 1.15 mg per ml hydrated sodium phosphate, 0.2 mg per ml potassium dihydrogen phosphate) was utilized to remove residual ammonium sulfate from the concentrated protein solutions (12). The resuspended proteins were transferred to a Spectral Por molecular porous membrane tubing with a pore size of 12000-14000 Kd and dialyzed against 500 volumes of phosphate buffered saline at 4°C for two to three hours. After this time, the dialysis sac was transferred into fresh phosphate buffered saline (500 volumes) at 4°C overnight. The dialyzed material was centrifuged (ten minutes, 4°C, 10000 X *g*) to remove any particulate matter.

Polyethylene glycol was used to further concentrate the inhibitor containing solution (12). The dialyzed solution was transferred to fresh dialysis membrane tubing (12000-14000 Kd cutoff) which was placed into a vessel containing polyethylene glycol (Carbowax® PEG 8000). Hydrated polyethylene glycol was replaced until the volume inside the membrane tubing was reduced to approximately one tenth of the original volume. After concentration, excess polyethylene glycol was removed by rinsing the membrane tubing in deionized water. The tubing was then dialyzed overnight against

1000 volumes of phosphate buffered saline at 4°C. The solution was centrifuged (ten minutes, 4°C, 10000 X *g*) to remove any remaining particulates and the supernatant retained.

Analysis of the concentrated protein solutions. After each step, the inhibitor containing solution was analyzed by the following procedures. First, samples from the concentration process were assayed by the well diffusion method, utilizing *M. smegmatis* and *M. luteus* ATCC 4698 as indicators, to determine whether or not the inhibitory activity was retained. The size of the zones of inhibition was measured in mm. and the titre of the inhibitor, in AU, was determined. Second, the total concentration of the protein solution, in mg per ml, was determined by 280 nm Protein Assay (12) or by the Bio-Rad 595 nm Protein Assay (Bradford Protein Assay). Bovine Serum Albumin diluted to various concentrations (10 µg per ml to 1500 µg per ml in deionized water) was utilized as the standard in the 280 nm Protein Assay. The absorbance of one ml of sample (test or standard) was measured at 280 nm. The spectophotometric readings of the standard were plotted against the known protein concentration utilizing the Microsoft® Excel X-Y Scatter graphics program. A regression line was formulated which was used to estimate the protein concentration of the test samples.

Bio-Rad 595 nm Protein Assay was also used to ascertained the protein concentration of the test samples. This procedure, performed according to manufacturer's instructional manual, utilized the Bio-Rad protein assay kit containing Bovine Serum Albumin as the standard. The samples were spectophotometrically measured at 595 nm and a regression line was formulated as described in the previous section.

Molecular weight approximation by membrane filtration. To approximate the

molecular weight of the inhibitory agent, the concentrated culture supernatant was filtered by centrifugation through membranes with various pore sizes (3K, 30K, 50K, 100K, Millipore Corp., Bedford, MA), according to manufacturer's instructions. The filtrates and retentates were assayed for inhibitory activity by the well diffusion method, utilizing *M. smegmatis* and *M. luteus* II, as the indicators. The approximate lower limit to the molecular weight range was determined to be the last filtrate possessing detectable inhibitory activity.

Treatment with heat, pH, and enzymes. Inhibitor preparations (crude extracts) in T-Soy broth, prepared as previously mentioned, were subjected to various treatments including heat, pH and digestion with enzymes. Subsequently, the treated samples were analyzed for anti-Mycobacterial activity by the well diffusion assay, utilizing *M. luteus* II, *M. phlei*, and *M. smegmatis* as indicators. The diameters of the zones of inhibition were measured in mm. to determine whether or not the treatment had any effect on the anti-Mycobacterial activity.

Heat stability was examined by boiling the sample for five minutes. The effect of pH on the inhibitory activity was investigated by subjecting samples to acidic and basic conditions. Acidic conditions were achieved by adding 1 M HCl until the sample reached a pH below 2. Basic conditions were achieved by adding 10 M NaOH. The following pH ranges were examined: pH 8 to pH 9, pH 9 to pH 10, pH 10 to pH 11, pH 11 to pH 12, and pH 12 to pH 13. After a fifteen minute incubation at room temperature, the samples were neutralized. Samples were subjected to the following enzymatic treatment: proteinase-K, trypsin, chymotrypsinogen, lipase, protease p-6141, and protease p-5647. The enzyme-containing samples (1 mg per ml final concentration) were incubated for 30

minutes at 37°C. After incubation, the enzymes were inactivated by boiling the samples for five minutes.

Inhibitory spectrum of the inhibitory agent. In the spectrum studies, the producing strain was examined, as described previously, for antagonism towards various indicator strains, utilizing the deferred antagonism method. The indicator strains were incubated aerobically for 18 hours at 35°C. Inhibition was observed and recorded only as sensitive, referring to any growth inhibition displayed by the indicator strains, and resistant, indicating no inhibition of the indicators.

Inhibitor production on solid media and in liquid media by other *S. hominis* strains. Various procedures were performed to compare the inhibitory agent production of *S. hominis* strain αM to other strains of *S. hominis*. To determine whether or not this inhibitory activity is unique to *S. hominis* strain αM, the deferred antagonism method and the well diffusion method were performed. Several *S. hominis* strains, *S. hominis* ATCC 27845, *S. hominis* ATCC 27847, *S. hominis* E56A-2, and *S. hominis* E36B-4, were examined for the presence of inhibitory activity and then the inhibitory activity was compared to the activity exhibited by *S. hominis* strain αM. *M. smegmatis*, *M. luteus* I1, and the producer strains were utilized as indicator strains. For the deferred antagonism method, inhibition was observed and recorded as ++ (strong inhibition), + (weak inhibition) or - (resistant). For the well diffusion method, the crude extracts were prepared as previously described in the section on concentration procedures. Crude extracts from *S. hominis* strain αM and the other *S. hominis* strains were assayed for the presence of inhibitory activity towards the indicator strains. The zones of inhibition, measured in mm., were recorded.

Time of production of the inhibitory agent by *S. hominis* strain αM. The time of production of this inhibitory agent was ascertained by following cell growth and production of inhibitory activity directed against indicator strains. An overnight *S. hominis* strain αM culture was diluted 1000 fold into fresh THB and incubated in a 37°C shaking water bath. Two ml samples were collected at 0, 2, and 4 hours and hourly thereafter for 12 hours. A final sample was obtained 24 hours after the initial inoculation. The following procedures were performed on the samples collected at various time intervals: determination of cell density at 600 nm. and assay for antimicrobial activity by the well diffusion assay, performed as described previously, with *M. luteus* ATCC 4698 and *M. smegmatis*, as the indicators. The data from these analyses were plotted utilizing the Microsoft® Excel X-Y Scatter graphics program.

Plasmid extraction. The technique utilized for the isolation of plasmid DNA was a modification of the Birnboim method (4). This method, used to determine plasmid possession in *S. hominis* strain αM and other *S. hominis* strains (27845, 27847, E36B-4, E54A-2), was performed as followed. Cultures, including the control culture, *S. epidermidis* 5, which typically containing five plasmids, pED501-pED505 (81), were inoculated into 5 ml Todd-Hewitt broth and incubated for six to eight hours at 37°C, 200 rpm. The cultures were centrifuged (one minute, RT, 14000 X g) and the pellet resuspended in GTE buffer (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA) containing 25 mg per ml lysozyme and 5 mg per ml lysostaphin (in TE buffer or deionized water). After a 30 minute incubation in a 37°C water bath, a 0.2 M NaOH/ 1% SDS solution was added to the suspension and incubated on ice for five minutes to allow cell lysis. Protein and chromosome was precipitated by the addition of 5 M potassium

acetate, pH 4.8. After five minutes on ice, the solution was centrifuged (three minutes, RT, 14000 X g). The supernatant was transferred to a microcentrifuge tube and one-half volumes of phenol and $CHCl_3$ isoamyl alcohol (24:1) were added. After centrifugation (ten minutes, RT, 14000 X g), the aqueous layer was transferred to a fresh microcentrifuge tube, two volumes of 95% ethanol added, and the solution left at -20°C overnight. The precipitated DNA was collected by centrifugation (ten minutes, RT, 14000 X g), washed twice with 70% ethanol, and dried. The pellet was resuspended in 25 µl TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) containing RNAse at a final concentration of 1 mg per ml.

Analysis of the plasmid extractions. Plasmid extracts were analyzed electrophoretically (200 V, one hour) on a 1% agarose gel with TBE buffer (Tris, boric acid, EDTA disodium salt). To maintain consistency, DNA purity and concentration were determined by UV spectophotometry (Shimadzo UV spectophotometer). Gels were examined using a UV transilluminator with the analysis carried out using the Kodak ID Scientific Imaging System and DC40 camera.

Plasmid curing in *S. hominis*. To examine the role that plasmids possessed by *S. hominis* strain αM play in the production and immunity of the inhibitory agent, the producer strain was treated with mitomycin C, rifampicin, and ethidium bromide. For the mitomycin C treatment, an overnight culture of *S. hominis* was inoculated into fresh THB to a 50-fold dilution and incubated to obtain logarithmic growth phase cells (three to five hours). To avoid build up the inhibitory agent in the media, the cells were washed twice utilizing the following procedure. First, the cells were centrifuged (one minute, RT, 14000 X *g*), and the supernatant removed. The pellet was resuspended in fresh THB

added to the original total volume. After the washings, the resuspended cells were treated with 0.5 µg per ml mitomycin C for 30 minutes at 37°C. The treated cells were centrifuged (one minute, RT, 14000 X *g*) and subsequently diluted with THB 10-fold to a final dilution of 10^{-10}, with 100 µl of each dilution plated onto TH agar plates. The plates were incubated at 35°C overnight and the isolated colonies screened for the loss of the ability to inhibit *M. smegmatis* and *M. luteus* I1.

The rifampicin treatment was performed essentially as described by Tagg and Wannamaker (100). An overnight *S. hominis* culture grown in BHI was inoculated into fresh BHI containing various concentrations of rifampicin to determine which concentration of rifampicin decreases the viable count by 10^2 to 10^3 fold as compared to an untreated culture. This concentration would be utilized for further curing experiments. The test culture was incubated for three to five hours, washed twice with fresh BHI by centrifugation (one minute, RT, 14000 X *g*) and the pellets resuspended in fresh BHI to a final volume of 5 ml. The resuspended pellets were diluted with BHI 10-fold to a final dilution of 10^{-5}. Each dilution was plated onto TH agar plates which were incubated at 35°C overnight. The isolated colonies were screened for the loss of the ability to inhibit *M. smegmatis* and *M. luteus* I1.

The ethidium bromide treatment was performed similarly to the rifampicin procedure with the following modifications (20). An overnight *S. hominis* strain αM culture grown in BHI was inoculated into fresh BHI containing various concentrations of ethidium bromide and incubated overnight to determine the last concentration of ethidium bromide to contain visible growth as compared to an untreated culture. This concentration would be utilized for further curing experiments. The ethidium bromide

treated culture was diluted with BHI 10-fold to a final dilution of 10^{-10}. Each dilution was plated onto BHI agar plates which were incubated at 35°C overnight. The isolated colonies were screened for the loss of the ability to inhibit *M. smegmatis* and *M. luteus* I1.

After curing treatments, screening for colonies not producing the anti-Mycobacterial agent was done by replicative stab inoculation. This was accomplished by the seeding of the indicator lawn onto a TH agar plate and transferring isolated colonies into a regular grid pattern on the seeded plate utilizing sterilized toothpicks. On each plate, sixty-four to one hundred individual colonies were screened for the loss of the ability to inhibit *M. smegmatis* and *M. luteus* I1 and a control plate was created to maintain colony isolation.

Since no isolated colonies were found to lack inhibitory activity, six to ten random colonies from each technique were examined by replicative stab inoculation, gram stain, Birnboim plasmid extraction, and catalase test to determine if the colonies were *S. hominis*.

CHAPTER III

RESULTS

Production of anti-Mycobacterial activity on solid media as detected by the deferred antagonism method. *S. hominis* strain αM strongly inhibited the growth of *M. phlei* and *M. smegmatis* on all the solid media tested (Table 6). *S. epidermidis* strain 5, a closely related Staphylococcus, failed to inhibit either *Mycobacterium* species. In addition, *S. epidermidis* strain 5 strongly inhibited *S. hominis* strain αM on all the solid media examined, while *S. hominis* strain αM inhibited *S. epidermidis* strain 5 on TH agar and showed weak inhibition on T-Soy agar. Furthermore, both *S. epidermidis* strain 5 and *S. hominis* strain αM were resistant to their own inhibitory activity. Both *S. hominis* strain αM and *S. epidermidis* strain 5 inhibited *M. luteus* I1, however, the degree of inhibition varied depending on the media. *S. hominis* strain αM strongly inhibited *M. luteus* I1 on all the solid media tested. *S. epidermidis* strain 5 strongly inhibited *M. luteus* Il on TH, T-Soy, and BHI agars, with only weak inhibition detected on CBAB agar. No inhibitory activity was detected against *M. luteus* I1 on BA agar.

Production of the anti-Mycobacterial activity in liquid media. To facilitate further studies of the anti-Mycobacterial agent, the production of the inhibitory agent was examined in liquid media. Since *S. hominis* strain αM displayed strong inhibitory activity when grown on TH agar plate, the broth media was chosen to examine the optimal conditions for maximum production. The conditions for anti-Mycobacterial agent production in THB by *S. hominis* strain αM chosen as the standard conditions was incubation at 37°C in a shaking water bath (Table 7). No detectable inhibitory activity

TABLE 6. *S. hominis* strain αM anti-Mycobacterial activity on various solid media

	Media[a]	TH		CBAB		T-Soy		BHI		BA	
Indicator[b]	Producer[c]	SH	#5	SH	#5	SH	#5	SH	#5	SH	#5
M. luteus II		++[d]	++	++	+/-	++	++	++	++	++	-
M. phlei		++	-	++	-	++	-	++	-	++	-
M. smegmatis		++	-	++	-	++	-	++	-	++	-
S. hominis αM		-	++	-	++	-	++	-	++	-	++
S. epidermidis 5		++	-	-	-	+	-	-	-	-	-

[a] Anti-Mycobacterial activity was examined by deferred antagonism method on various solid media such as TH (Todd-Hewitt), CBAB (Columbia Blood Agar Base),T-Soy (Tryptic Soy), BHI (Brain Heart Infusion), and BA (Blood Agar).

[b] Indicator organisms included *Micrococcus luteus* 11, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *S. hominis* strain αM, and *S. epidermidis* strain 5.

[c] Producer strains were *S. hominis* strain αM (SH) and *S. epidermidis* strain 5 (#5).

[d] The degree of inhibition was measured by the following scale : strong inhibition is represented by ++, weak inhibition is represented by +, partial inhibition with colony growth within the area of the original strip is represented by +/-, and no inhibition is represented by -.

TABLE 7. Aeration conditions for maximum inhibitory activity production

Condition[a]	Diameter of zone (mm.)
Non-shaking	
Candle Jar at 32°C	-
Aerobically at 32°C	-
Aerobically at 37°C	-
Aerobically at 55°C	-
Anaerobically at 35°C	-
Shaking	
Water Bath at 37°C	11

[a] The inhibitory activity of *S. hominis* strain αM was examined by varying the growing conditions. *S. hominis* strain αM was inoculated into THB, incubated for 24 hours, and assayed by the well diffusion method for the presence of inhibitory activity towards *M. smegmatis*.

towards *M. smegmatis* was observed with any of the other incubation conditions tested. Various other liquid media were examined for their ability to support inhibitor production under the standard incubation conditions. The inhibitory activity expressed by *S. hominis* strain αM was detected in all the culture supernatants, utilizing the indicators, *M. luteus* II and *M. phlei*, with the size of the inhibitory zone increasing as the volume of culture supernatant added to the wells was increased. (Table 8, Figs. 1 and 2). No activity against *M. smegmatis* was detected when 20 µl of BHI and TH + YE culture supernatants were added to the wells, however, inhibition was observed when 60 µl of the culture supernatant was used. No inhibitory activity against *M. smegmatis* and *M. phlei* was detected in the culture supernatants from *S. epidermidis* strain 5. Of the two broths that showed the most inhibitory activity, THB was chosen as the media for further inhibitory agent studies, although, T-Soy broth was utilized for testing the effects of heat, pH, and enzymes on the inhibitor.

Concentration of the inhibitory agent. The inhibitory agent was concentrated from the culture supernatant by two alternative methods of ammonium sulfate precipitation: step-wise precipitation and one-step precipitation. Prior to ammonium sulfate precipitation, *S. hominis* strain αM was grown in THB and incubated in a 37°C shaking water bath for 18 hours. Upon step-wise precipitation of the crude extract, the maximum amount of total activity (in AU), recovered at 50% saturation, was 32 when the indicator, *M. luteus* ATCC 4698, and 16 when the indicator, *M. smegmatis*, was used (Table 9). A decrease in the amount of activity was observed as the saturation of ammonium sulfate was increased, with little or no activity at 70% and 80% saturation, respectively. This pattern was observed when either indicator was used.

TABLE 8. *S. hominis* strain αM anti-Mycobacterial activity in various liquid media

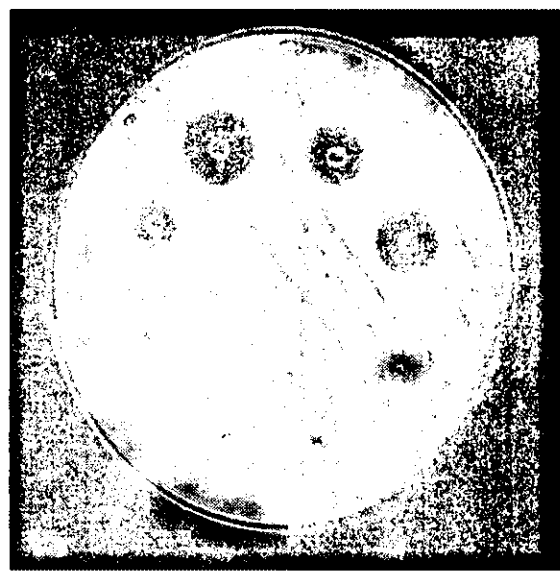
	SH[a, b]				#5[a, b]			
Indicator[c]	THB	BHI	TH +YE	T-Soy	THB	BHI	TH +YE	T-Soy
Well vol. 20 µl								
M. luteus II	14	9	10	12	-	-	-	-
M smegmatis	10	-	-	8	-	-	-	-
M phlei	13	9	9	12	-	-	-	-
Well vol. 60 µl								
M. luteus II	18	14	15	17	-	-	-	-
M smegmatis	18	11	12	17	-	-	-	-
M phlei	17	11	11	14	-	-	-	-

[a] *S. hominis* strain αM (SH) and *S. epidermidis* strain 5 (#5) were examined for the production of anti-Mycobacterial activity in a liquid broth by a modified version of the well diffusion method, using volumes of 20 µl and 60 µl of the culture supernatant. This activity was measured as the diameter of the zone in mm.. No inhibition is represented by -.

[b] The inhibitory activity was examined in various liquid media such as TH + YE (Todd-Hewitt broth with Yeast Extract), T-Soy (Tryptic Soy broth), BHI (Brain Heart Infusion), and THB (Todd-Hewitt broth).

[c] The indicator organisms include *Micrococcus luteus* II, *Mycobacterium smegmatis*, and *Mycobacterium phlei.*

A B



C

FIG. 1. *S. hominis* strain αM (αM) and *S. epidermidis* strain *5* (#5) examined for the production of anti-Mycobacterial activity in a liquid broth by a modified version of the well diffusion method using 20 µl of the culture supernatant. The inhibitory activity was examined in the following liquid media: BHI (Brain Heart Infusion),THB (Todd-Hewitt broth),TH + YE (Todd-Hewitt broth with Yeast Extract), and T-Soy (Tryptic Soy broth). The wells were arranged on the agar plates as followed, starting from the top and going clockwise: αM TH + YE, αM T-Soy, #5 T-Soy, #5 TH + YE, #5 THB, #5 BHI, αM BHI and αM THB. The indicator organisms utilized include *M. phlei* (A), *M. smegmatis* (B),and *M. luteus* II (C). Note inhibition zones for all three indicators for αM THB and αM T-Soy. However, for the indicator organisms *M. phlei* (A) and *M.luteus* II (C), additional zones were observed for *S. hominis* strain αM grown in BHI and TH + YE, which was not observed for the *M. smegmatis* indicator (B). No zones were observed for any of the liquid broths inoculated with *S. epidermidis* strain 5. This activity was measured as the diameter of the zone in mm..

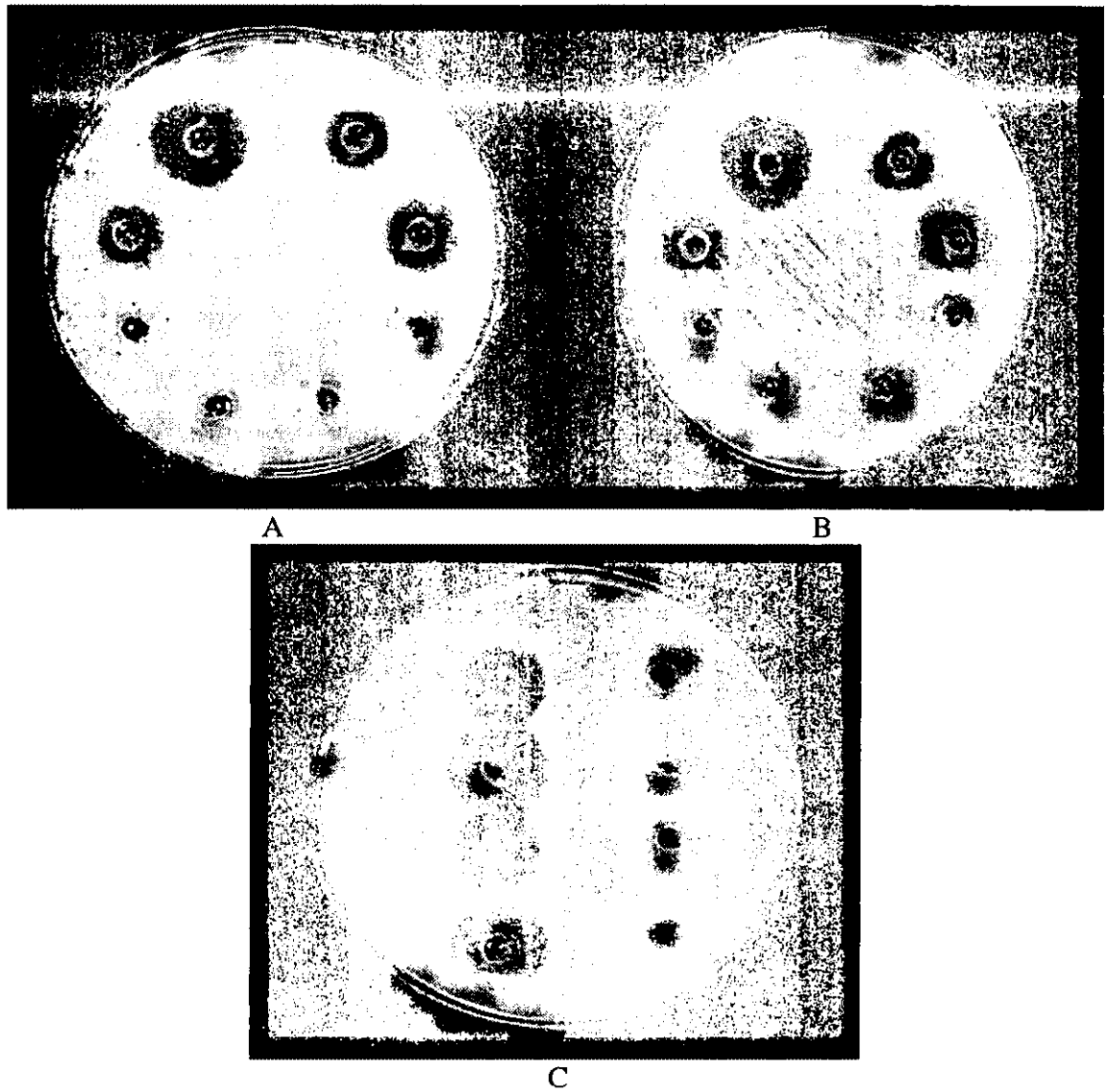


FIG. 2. *S. hominis* strain αM (αM) and *S. epidermidis* strain 5 (#5) examined for the production of anti-Mycobacterial activity in a liquid broth by a modified version of the well diffusion method using 60 µl of the culture supernatant. The inhibitory activity was examined in the following liquid media: BHI (Brain Heart Infusion),THB (Todd-Hewitt broth),TH + YE (Todd-Hewitt broth with Yeast Extract), and T-Soy (Tryptic Soy broth). The indicator organisms utilized include *M. phlei* (A), *M. smegmatis* (B),and *M. luteus* II (C). The samples on the agar plates seeded with the *Mycobacterium* species (A & B) were arranged as followed, starting from the top and going clockwise: αM TH + YE,αM T-Soy, #5 T-Soy, #5 TH + YE, #5 THB, #5 BHI, αM BHI and αM THB. The samples on the agar plates seeded with the *M. luteus* II (C) were arranged as followed, starting from the top and going across: αM BHI, #5 BHI, αM THB, #5 THB, αM TH + YE, #5 TH + YE, αM T-Soy, and #5 T-Soy. Note inhibition zones for all three indicators for all the liquid media inoculated with *S. hominis* strain αM. No zones were observed for any of the liquid broths inoculated with *S. epidermidis* strain 5. The inhibitory activity was measured as the diameter of the zone in mm..

TABLE 9. The zones of inhibition, the arbitrary unit (AU) titred, the specific activity, and the total protein concentration recovered after step-wise and one-step ammonium sulfate precipitation and polyethylene glycol concentration of the culture supernatant of *S. hominis* strain αM

Samples	*M. luteus*			*M. smegmatis*			
	Diameter of zone (mm.)[a]	Total activity (AU)[b]	Specific activity (AU per mg)	Diameter of zone (mm.)[a]	Total activity (AU)[b]	Specific activity (AU per mg)	Total protein conc.[c] (mg per ml)
Culture Supernatant[d]	26	16	141.6	12	1	8.8	0.113
Step 50% Pellet[e]	30	32	48.0	21	16	24.0	0.666
Step 60% Pellet[e]	16	16	47.9	16	2	6.0	0.334
Step 70% Pellet[e]	10	1	7.7	14	1	7.7	0.130
Step 80% Pellet[e]	-	-	-	10	1	-	n/d[g]
Culture Supernatant[d]	26	16	141.6	12	1	8.8	0.113
AS Pellet[f]	13	128	184.7	10	1	1.4	0.693
D Supernatant[f]	29	64	232.7	9	1	3.6	0.275
PEG Supernatant[f]	n/t	> 128	168.0	13	1	1.3	0.762
PEG-D Supernatant[f]	32	> 128	208.8	13	1	1.6	0.613

[a] The undiluted samples were assayed by the well diffusion method to detect inhibitory activity of *S. hominis* strain αM and the inhibition zones were measured in mm. as detected by the indicator organisms, *M. luteus* ATCC 4698 and *M. smegmatis*.

[b] Represents the last two fold dilution that exhibits inhibitory activity.

[c] The approximate amount of protein in mg per ml of each step was determined by Bio-Rad 595 nm protein assay from a regression line of $y = 0.9004x + 0.43$, $R^2 = 0.9949$.

[d] From an overnight culture of *S. hominis* strain αM grown in THB and incubated shaking at 37°C.

[e] The step-wise ammonium sulfate procedure was performed on the culture supernatant.

[f] Ammonium sulfate was added to the culture supernatant to a final percentage of 80%. The precipitated proteins were resuspended (AS) and dialyzed in phosphate buffered saline (D). The dialyzed solution containing the precipitated proteins was concentrated by polyethylene glycol (PEG) and dialyzed to remove excess PEG (PEG-D).

[g] Protein was not detected in this sample.

Protein concentration, in mg per ml, and the specific activity, in AU per mg, for the most part, followed the same pattern as that of activity recovery. The maximum protein concentration and the maximum specific activity in the ammonium sulfate precipitated material was recovered at 50% saturation. The maximum protein concentration was determined by the Bio-Rad 595 nm protein assay to be 0.666 mg per ml. The maximum specific activity recovered was ascertained to be 48 AU per mg, as detected by the indicator, *M. luteus* ATCC 4698, and 24 AU per mg as detected by the indicator, *M. smegmatis*.

Ammonium sulfate precipitation and polyethylene glycol concentration were performed on the culture supernatant to determine the effect these treatments would have on the inhibitory agent's total activity, specific activity, and protein concentration. To remove the maximum amount of inhibitory agent from the culture supernatant, a one-step ammonium sulfate precipitation to a final saturation of 80% was utilized. Compared to the crude extract, the ammonium sulfate precipitated material resulted in a 8-fold increase in the total activity and the polyethylene glycol treated material resulted in a higher than 8-fold increase in the total activity, as detected by the indicator, *M. luteus* ATCC 4698. The maximum inhibitory activity was observed when the polyethylene glycol concentrated protein solution was dialyzed (>128 AU). The specific activity of the inhibitory agent increased after each treatment, as compared to the crude extract. When comparing the two treatments, the polyethylene glycol treated material lost specific activity, however, dialysis following each treatment improved the specific activity.

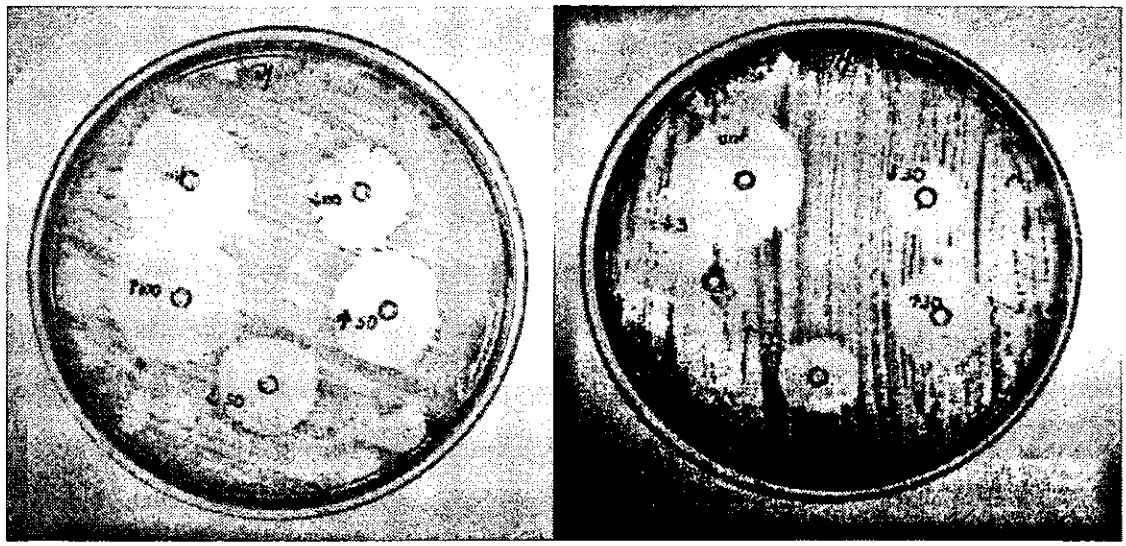
When the indicator, *M. luteus* ATCC 4698, was used, the maximum specific activity of the inhibitory agent occurred when the polyethylene glycol treated

material was dialyzed (237.7 AU per mg). Prior to ammonium sulfate precipitation, the Bio-Rad 595 nm protein assay detected a total protein concentration of 0.113 mg per ml in the culture supernatant (Table 9). The protein concentration increased five fold when the culture supernatant was concentrated with ammonium sulfate and six fold when the concentrated protein solution was further concentrated with polyethylene glycol.

Molecular weight approximation by membrane filtration. After concentrating the culture supernatant, the inhibitory agent produced by *S. hominis* strain αM was filtered through membranes of various pore sizes to gain information about the approximate molecular weight. When utilizing *M. luteus* II as the indicator, a molecular weight of 12,000-30,000 MW was indicated.

The inhibitory activity towards the more agent-sensitive indicator, *M. luteus* I1, was recovered in the retentates and filtrates collected after each filtration with activity last detected in the retentate of the membrane with a 3,000 MW cut off (Fig. 3B). However, when the indicator, *M. smegmatis*, was used, inhibitory activity was detected only up to the retentate collected after filtration with the membrane with a 50,000 MW cut off. When utilizing *M. luteus* II as the indicator, maximum inhibitory activity was observed in the retentate collected after filtration with the membrane with the pore size of 100,000 MW. The activity decreased as filtered, but consistently remained in higher amounts in the retentate than the filtrate after each filtration because of further concentrating of the culture supernatant.

Treatment with heat, pH and enzyme digestion. A crude nonconcentrated extract of the inhibitory agent was subjected to various environmental conditions (Table 10). Inhibitory activity was retained when the preparation was boiled for five minutes,



A B

FIG. 3. Molecular weight approximation of the inhibitory agent by filtration of the concentrated supernatant through membranes of various pore size. The inhibitory activity was assayed by the well diffusion method with *M. luteus* II as the indicator. (A) The filtrates and retentates are as follow going clockwise: the filtrate containing molecules smaller than 100,000 MW, the retentate containing molecules ranging between 50,000-100,000 MW, the filtrate containing molecules smaller than 50,000 MW, the retentate containing molecules larger than 100,000 MW, and the unfiltered dialyzed, polyethylene glycol treated protein solution (positive control). (B) The filtrates and retentates are as follow, going clockwise: the filtrate containing molecules smaller than 30,000 MW, the retentate containing molecules ranging between 30,000-50,000 MW, the retentate containing molecules ranging between 3,000-30,000 MW, the filtrate containing molecules smaller than 3,000 MW, and the unfiltered dialyzed, polyethylene glycol treated protein solution (positive control). Inhibitory activity was observed for all filtrates except the filtrate that contained molecules smaller than 3,000 MW (B).

TABLE 10. Physical and chemical characteristics of the inhibitory activity produced by *S. hominis* strain αM

Treatment [a]	*M. luteus* 11 [b]	*M. phlei* [b]	*M. smegmatis* [b]
Untreated Culture Supernatant	26	22	19
Boiling [c]	25	23	15
pH < 2 [d]	25	27	17
pH 8 to pH 9 [d]	26	24	18
pH 9 to pH 10 [d]	26	22	18
pH 10 to pH 11 [d]	25	23	18
pH 11 to pH 12 [d]	17	19	12
pH 12 to pH 13 [d]	15	-	-
Proteinase K [e]	-	-	-
Chymotrypsin [e]	25	20	17
Trypsin [e]	-	-	-
Protease [e]	-	-	-

[a] The culture supernatant of *S. hominis* strain αM was subjected to various treatments.

[b] The inhibitory activity was assayed by the well diffusion method (zones measured in mm.) utilizing the following indicator organisms: *M. luteus* 11, *M. phlei* and *M. smegmatis*.

[c] The culture supernatant was heated at 100°C for five minutes.

[d] pH treatment was performed by adding HCl or NaOH until the desired pH was reached. The solution was incubated for 15 minutes and neutralized by the addition of either HCl or NaOH.

[e] The enzyme-containing samples (1 mg per ml final concentration) were incubated for 30 minutes at 37°C and inactivated by boiling the samples for five minutes.

treated with acid (HCl) to lower the pH to less than two, or treated enzymatically with chymotrypsin. However, inhibitory activity decreased following alkali treatment above pH 8 and continued to decrease until complete absence of activity was detected at a pH greater than twelve for both the *Mycobacterium* indicators. *M. luteus* II still detected some inhibitory activity following treatment at a high pH. Enzymatic treatment with Proteinase K, trypsin, and protease resulted in a loss of inhibitory activity.

Inhibitory spectrum of the inhibitory agent. Various microorganisms were examined for susceptibility to the inhibitory agent of the producer strain by the deferred antagonism method. Antagonism was observed against all the gram-positive organisms tested, except for *Erysipelothrix rhusiopathiae*, while all the tested gram-negative organisms were resistant (Table 11). The two yeast genera, *Saccharomyces* and *Candida*, were also resistant to the inhibitory agent.

Inhibitor production by other strains of *S. hominis*. Other *S. hominis* strains were examined for production of and susceptibility to the inhibitory agent produced by *S. hominis* strain αM on solid and in liquid media. On TH agar, *S. hominis* strain αM strongly inhibited the following *S. hominis* strains: 27845, 27847, and E56A-2, while E36B-4 showed less sensitivity (Fig. 4). *S. hominis* strain αM was resistant to its own inhibitory action. The inhibitory activity produced by other strains of *S. hominis* on a solid media was also examined (Table 12). *S. hominis* strains, 27845, 27847, and E56A-2, did not inhibit any of the indicators (Fig. 5A). However, *S. hominis* strain E36B-4 strongly inhibited 27847 and weakly inhibited *S. hominis* strain αM and E56A-2 (Fig. 5B). *S. hominis* strain E36B-4 did not inhibit the growth of itself, 27845, *S. epidermidis* 5, *M. luteus* II, or *M. smegmatis*.

TABLE 11. The susceptibility of various organisms towards *S. hominis* strain αM inhibitory activity[a]

Susceptible Organisms[b]	Resistant Organisms[b]
Gram-positive Cocci	Gram-negative Rods
Staphylococcus aureus ATCC 25823	*Enterobacter faecalis* 19433
S. epidermidis ATCC 12228	*Proteus vulgaris* ATCC 13315
Micrococcus luteus ATCC 4698	*P. mirabilis* ATCC 131
Enterococcus faecalis ATCC 19433	*Salmonella typhimurium* ATCC 14024
Aerococcus viridans	*Serratia marcescens* ATCC 13048
Streptococcus bovis	*Escherichia coli* ATCC 25922
S. pneumoniae	*Shigella sonnei* ATCC 25931
S. salivarius	*S. flexneri* ATCC 12222
S. mutans	*Vibrio alginolyticus* 253
S. pyogenes ATCC 19615	*V. parahaemolyticus*
Group B Streptococci	*Pseudomonas aeruginosa* ATCC 27853
Group C Streptococci	*P. denitrificans* 297
Group F Streptococci	*Alcaligenes faecalis* 104
Group G Streptococci	*Klebsiella pneumoniae* ATCC 13883
Gram-positive Rods	Gram-negative Diplococci
Bacillus subtilis ATCC 6051	*Branhamella catarrhalis*
B. subtilis ATCC 6054	
B. cereus ATCC 14579	Gram-positive Rods
B. stearothermophilus ATCC 12980	*Erysipelothrix rhusiopathiae*
Corynebacterium kutscheri ATCC 15677	
C. xerosis ATCC 373	Yeast
Mycobacterium phlei 188	*Saccharomyces cerevisiae*
M. smegmatis	*Candida albicans*

[a] Determination of the susceptibility of the organisms to the inhibitory agent produced by *S. hominis* strain αM were based on the deferred antagonism assay.
[b] Organisms obtained from the culture collection of the Biological Sciences Support Faculty, Old Dominion University, Norfolk, Va..

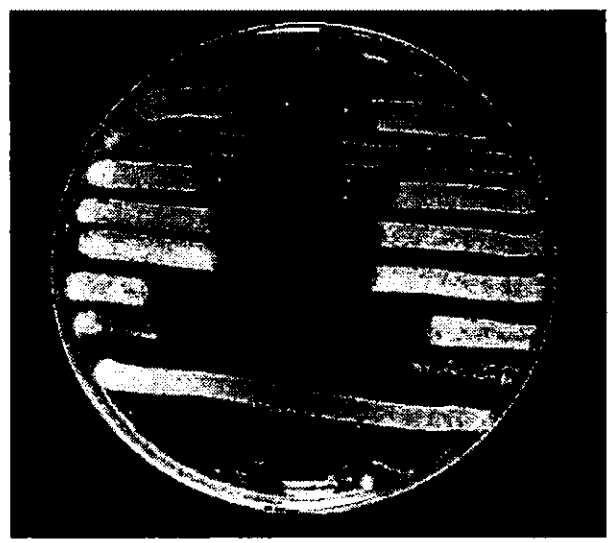


FIG. 4. The inhibitory activity of *S. hominis* strain αM towards various *S. hominis* strains as examined by the deferred antagonism method. The indicator organisms used were streaked perpendicular to the initial producer strain streak. From top to bottom: *S. epidermidis* 5, *S. hominis* E36B-4, *S. hominis* E56A-2, *S. hominis* 27845, *S. hominis* 27847, *M. luteus* 11, *M. smegmatis*, and *S. hominis* strain αM.

TABLE 12. Inhibitor production on Todd-Hewitt agar by various *S. hominis* strains[a]

	Producers				
Indicators[b]	αM	E36B-4	27845	27847	E56A-2
αM	-	+	-	-	-
E36B-4	+	-	-	-	-
E56A-2	++	+	-	-	-
27845	++	-	-	-	-
27847	++	++	-	-	-
II	++	-	-	-	-
MS	++	-	-	-	-
#5	++	-	-	-	-

[a] Inhibitor production was assayed by the deferred antagonism method. The degree of inhibition was measured by the following scale : ++ represents strong inhibition, + represents weak inhibition, and - represents resistance.

[b] The indicator strains were as follow : αM (*S. hominis* strain αM), E36B-4 (*S. hominis* strain E36B-4), E56A-2 (*S. hominis* strain E56A-2), 27845 (*S. hominis* strain 27845), 27847 (*S. hominis* strain 27847), II (*M. luteus* II), MS (*M. smegmatis*), and #5 (*S. epidermidis* strain 5).

A B

FIG. 5. The inhibitory activities of *S. hominis* strains 27847 (A) and E36B-4 (B) towards various *S. hominis* strains as examined by the deferred antagonism method. The following organisms were streaked perpendicular to the initial producer strain streak, from top to bottom: *S. epidermidis* 5, *S. hominis* E36B-4, *S. hominis* E56A-2, *S. hominis* 27845, *S. hominis* 27847, *M. luteus* 11, *M. smegmatis*, and *S. hominis* strain αM. Note that *S. hominis* strains 27847 (A) did not inhibit *M. smegmatis*. although this was not clear on the photograph.

The crude culture supernatant of *S. hominis* strain αM was compared to other *S. hominis* supernatants for inhibitory activity by the well diffusion method utilizing *M. smegmatis*, *M. phlei* and *M. luteus* II as the indicators (Table 13). For all indicators, only *S. hominis* strain αM exhibited inhibition activity.

Growth of *S. hominis* and the production of the inhibitory agent. Inhibitor production was followed at various points through the growth of *S. hominis* strain αM (Fig. 6). Under the conditions tested, *S. hominis* strain αM shows a two-hour lag phase with an approximately four hour logarithmic phase, as determined by the 600 nm. spectophotometric readings. The inhibitory agent activity, as assayed by the well diffusion method utilizing *M. luteus* ATCC 4698 as the indicator, was produced in mid-logarithmic phase. The production of activity against *M. smegmatis* was detected later in growth (after seven hours).

Plasmid possession. The producer strain, *S. hominis* strain αM, was ascertained to contain a number of plasmids by the presence of up to seven bands when the preparations extracted by the Birnboim method were ran on a 1% agarose gel with TBE buffer (Fig. 7). Plasmid possession of the other strains of *S. hominis* was examined (Fig. 7). The other *S. hominis* strains varied in band number; *S. hominis* 27845 was shown to have four bands. Both *S. hominis* E36B-4 and *S. hominis* E56A-2 potentially contain 5 plasmids, while *S. hominis* 27847 may have as many as eight plasmids. *S. epidermidis* 5 resulted in five bands.

The role of the plasmid in the inhibitory activity production. To determine the role of the plasmid in the inhibitory activity production, *S. hominis* αM was treated with various curing agents. The appropriate concentrations of the curing agents were

TABLE 13. Inhibitor production in Todd-Hewitt broth by various *S. hominis* strains[a]

Producers	Indicators		
	M. phlei	*M. smegmatis*	*M. luteus* II
S. hominis αM	16	14	20
S. hominis E36B-4	-	-	-
S. hominis E56A-2	-	-	-
S. hominis 27845	-	-	-
S. hominis 27847	-	-	-
S. epidermidis 5	-	-	-

[a] Inhibitor production was assayed on Todd-Hewitt agar by the well diffusion method utilizing 60 µl of the culture supernatant per well. Inhibitory activity was measured as the diameter of the zone in mm.. No inhibition was represented by -.

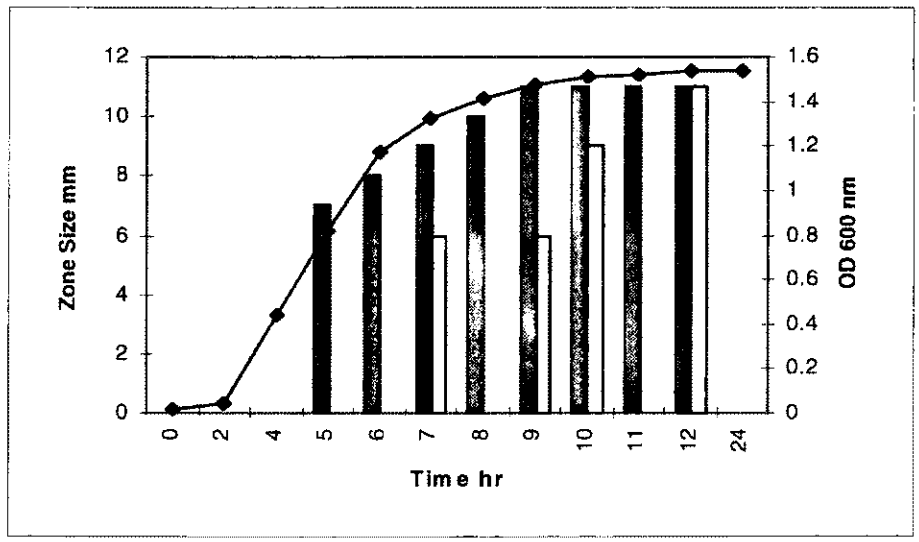


FIG. 6. Growth of *S. hominis* strain αM and time of production of the inhibitory agent. The growth of *S. hominis* strain αM, as a measure of the cell density, was determined by spectophotometry readings at 600 nm. recorded at various times. The agent production time was ascertained as a measure of the diameter of the zone of inhibition in mm. resulting from the activity assay in a liquid broth utilizing *M. luteus* ATCC 4698 (black bar) and *M. smegmatis* (white bar) as indicators.

A B C D E F G

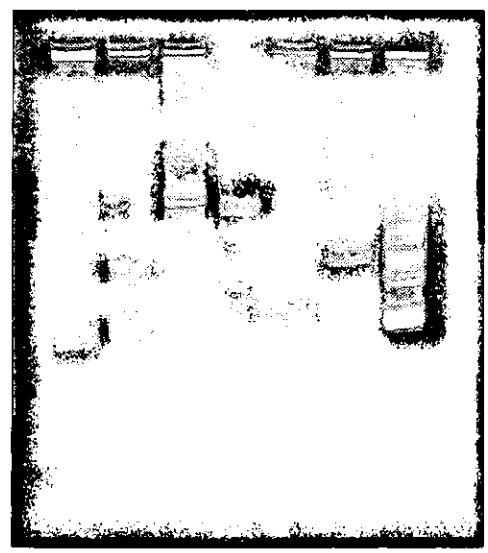


FIG. 7. Agarose gel following plasmid extractions of *S. hominis* strain αM, other *S. hominis* strains, and *S. epidermidis* strain 5, a staphylococcus containing 5 plasmids. The lane assignments and number of bands are as followed: A, *S. hominis* E36B-4 (5 bands); B, *S. hominis* E56A-2 (5 bands); C, *S. epidermidis* 5 (5 bands); D, λ-Sty molecular weight marker (8 bands); E, *S. hominis* stain αM (7 bands); F, *S. hominis* 27847 (8 bands), and G, *S. hominis* 27845 (4 bands). Note that some of the bands that were visible on the gel did not photograph.

ascertained to ensure curing of *S. hominis* αM plasmids; The concentration of rifampicin that decreased the viable count by 10^2-10^3 was ascertained to be 0.02 μg per ml and the last concentration of ethidium bromide that contained visible growth was 18.75 μg per ml. The concentration of mitomycin C utilized in the curing experiment was the concentration used in previous studies (95). Treatment with mitomycin C, rifampicin, or ethidium bromide failed to yield colonies that no longer produced the agent (Table 14). To ascertain whether or not treated colonies were *S. hominis* αM, ten random treated cells (ten of rifampicin, ten of ethidium bromide) were examined. It was determined that the colonies were catalase-positive, Gram-positive cocci. Plasmid extractions performed on treated cells showed band patterns that were similar but not always identical to that of *S. hominis* αM, the parent producer strain (Fig. 8).

TABLE 14. Curing treatments on *S. hominis* strain αM

Treatment	Concentration (μg per ml)	Total Colonies Screened [a]	No. of Non-producing Strains [b]
Mitomycin C	0.05	692	0
Rifampicin	0.02	100	0
Ethidium Bromide	18.75	1000	0

[a] Colonies screened by replicative stab inoculation with *M. smegmatis* and *M. luteus* II as the indicators.
[b] Colonies exhibiting no inhibition towards either indicator.

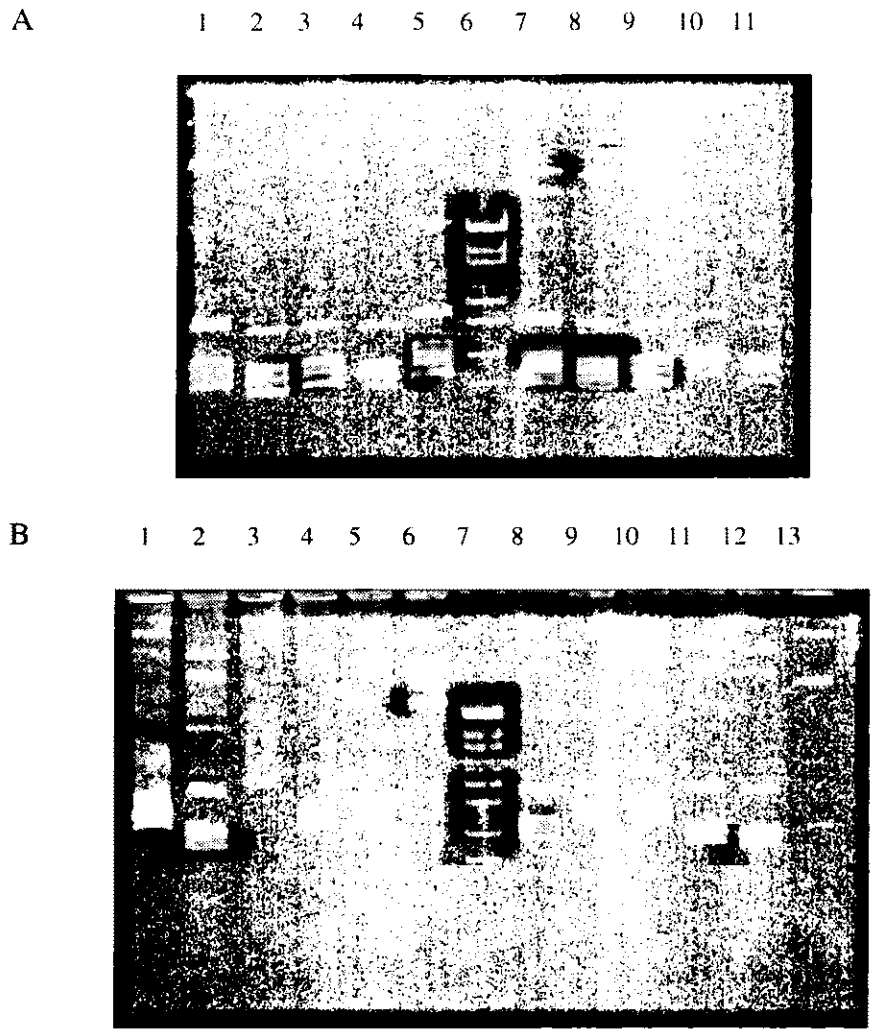


FIG. 8. Plasmid extractions of *S. hominis* strain αM, *S. hominis* strains treated with ethidium bromide (A) and rifampicin (B). The lane assignments are as follows. A: Lanes 1, 2, 3, 4, and 5 are ethidium bromide treated *S. hominis* strain αM colonies 1-5; Lane 6, λ-Sty molecular weight marker; Lanes 7, 8, 9, 10, and 11 are ethidium bromide treated *S. hominis* strain αM colonies 6-10. B: Lanes 1, 2, 3, 4, 5, and 6 are rifampicin treated *S. hominis* strain αM colonies 1-6; Lane 7, λ-Sty molecular weight marker; Lanes 8, 9, 10, 11, 12 and 13 are rifampicin treated *S. hominis* strain αM colonies 7-12.

CHAPTER IV

DISCUSSION

Though the production of bacteriocins and other antimicrobial agents by staphylococci has been reported, this is the first known account of an inhibitory agent produced by a strain of *S. hominis*.

S. hominis αM produces an antimicrobial activity against *Mycobacterium* spp., an activity which is unique for antimicrobial agents produced by staphylococci. Anti-Mycobacterial activity has been detected in *Streptococci* spp. and *Lactococcus* spp., including the lantibiotics salivaricin A, produced by *Streptococcus salivarius* (83), and nisin (41), produced by *Lactococcus lactis* spp., respectively. This anti-Mycobacterial activity was detected on various solid and liquid media. Because production of the inhibitory agent occurred in liquid media, characterization and concentration of the agent produced by *S. hominis* αM could be accomplished in a simplified manner. Similarly, inhibitory agent production in liquid media is observed for other gram-positive bacteria, including *Staphylococci* spp. [the lantibiotic Pep5 (87) and the bacteriocin D91 (52)], *Bacillus* spp. [the lantibiotic mersacidin (18) and the bacteriocin cerein (73)], *Lactococcus* spp. [the lantibiotic nisin (45)], and *Lactobacillus* spp. [the bacteriocins plantaricin S and T (48)]. However, not all bacteriocins of gram-positive bacteria are produced in greater quantities in a liquid media. For example, the production of various streptococcins, Sal P by *Streptococcus salivarius* strain P and salivaricin A, appears to be increased in solid media rather than liquid media (83, 98).

The inhibitory agent produced by *S. hominis* αM was characterized has possessing

protease, proteinase K, and trypsin treatments. This finding was consistent with other bacteriocins of gram-positive bacteria. The proteolytic enzyme, chymotrypsin did not effect the activity of the inhibitory agent. Therefore, it is possible that this agent lacks bulky hydrophobic residues or that the cleavage of the sites containing these residues does not effect inhibitory activity. Although the agent produced by *S. hominis* αM was inactivated when treated with lipase (data not shown), the inactivation was probably caused by protease contamination of the stock lipase mixture. There are bacteriocins of gram-positive bacteria that have been found to contain a lipidic moiety. One example includes the bacteriocin plantaricin S, which was inactivated by lipase as well as trypsin, chymotrypsin, proteinase and other hydrolytic enzymes (48).

Besides containing an active protein component, the inhibitory agent is heat-stable, which is a characteristic common to other bacteriocins and antimicrobial agents produced by Staphylococci such as the bacteriocins IYS2, Bac1829, and Pep5 (68, 66, 43), as well as bacteriocins produced by other gram-positive bacteria. In addition to being stable in heat, this agent was determined to be acid-stable with the stability of the agent decreased as the pH was increased. Loss of activity against *Mycobacterium* spp. occurred at a pH greater than 12. These finding are similar to other staphylococcal antimicrobial agents although more stable than many agents at basic pH. For example, the lantibiotic Pep5 (63) and the bacteriocin D91 (52) have activity in the range of pH 2 to pH 8 and pH 1.5 to pH 8, respectively. In contrast, the bacteriocin plantaricin C19, produced by *Lactobacillus plantarum* C19, is only stable at pH 2 to pH 6 (3) and the bacteriocin cerein, produced by *Bacillus cereus* GN105, is active over a large range with partial sensitivity to extreme acidic and basic conditions (71).

As is observed with other bacteriocins produced by Staphylococci and other gram-positive organisms, this agent exhibited a broad spectrum of activity with varying degrees of specific activity towards gram-positive bacteria, including other *S. hominis* strains, and not gram-negative organisms (66). For example, the bacteriocin IYS2, produced by *S. aureus* IYS2, has a broad spectrum of activity against oral indigenous bacteria including *Streptococcus salivarius*, *Propionibacterium acnes*, *Corynebacterium parvulum*, *Actinomyces israelii*, as well as several *S. aureus* strains. The bacteriocin plantaricin S has a broad spectrum of activity which includes gram-positive organisms such as *Enterococcus faecalis*, *Propionibacterium* spp., *Clostridium* spp., *Micrococcus* spp., *Leuconostoc* spp., *Lactococcus* spp., and *Lactobacillus* spp.. The spectrum of activity of the inhibitory agent produced by *S. hominis* αM is similar to that of the lantibiotic nisin, which inhibits Staphylococci, Streptococci, Bacilli, and Mycobacteria (45). However, some Staphylococci do inhibit gram-negative organisms. The inhibitory agent, D91, inhibits various *S. aureus* strains, Group A Streptococci, as well as the gram-negative organisms, *Proteus* spp., *E. coli*, and *Pseudomonas* spp. (52). The inhibitory agent, Bac1829, inhibits *S. aureus* RN4220, *Corynebacterium* spp., *Bordetella* spp., and the gram-negative organisms, *E. coli*, *Salmonella typhimurium*, *Haemophilus* spp., and *Actinobacillus* spp. (21). Some bacteriocins of gram-positive organisms inhibit only a narrow spectrum including the bacteriocin lactacin B which only inhibits Lactobacilli (7), the lantibiotic Pep5 which only inhibits *S. aureus* and coagulase-negative Staphylococci and Micrococci (65), and the bacteriocin cerein which only inhibits *Bacillus cereus* (71). In the course of this study, two *M. luteus* strains, *M. luteus* I1 and *M. luteus* ATCC 4698, were determined to be more sensitive to the inhibitory agent than *Mycobacterium* spp..

Because of this and the more rapid growth of *M. luteus* spp. (24 hour growth), it was decided to utilized this bacterium, along with *M. smegmatis*, as an indicator.

In this study, as well as the investigations of other bacteriocins produced by Staphylococci, ammonium sulfate precipitation was included as a step of the purification process. The maximum total activity, the maximum specific activities and the maximum protein concentrations of the agent produced by *S. hominis* αM were recovered when the crude extracts containing the inhibitory agent was precipitated with an ammonium sulfate saturation of 50%. In other studies, the saturation of ammonium sulfate used for purification ranged from 70% saturation for Bac1829 purification (66) to 90% saturation for Pep5 (43) and D91 (52) purification. However, purification of other bacteriocins of gram-positive organisms, such as lactacin F, use a much lower saturation (35% to 40%) to concentrate the inhibitory agent (70). Precipitating with ammonium sulfate and subsequent concentrating with polyethylene glycol on the culture supernatant increased the total activity and the specific activity as was expected. The indicator, *M. luteus* ATCC 4698, detected more activity than *M. smegmatis*, as was expected since *M. luteus* ATCC 4698 is more sensitive to the inhibitory agent.

The molecular weight range of the inhibitory agent was ascertained to be between 12000 to 30000 MW. Filtering the concentrated supernatant through various pore size membranes (Millipore) showed that activity was recoverable to the retentate of the 3000-cut off membrane, as detected by the indicator, *M. luteus* I1. Thus, approximating the molecular weight to be between 3000-30000. However, the lower limit was also determined by the presence of activity after the dialysis of the treated supernatant in membrane tubing with the pore size of 12000-14000 MW (Spectrum). These results

conflicted with the findings when utilizing the other indicator, *M. smegmatis*, which detected activity only up to the retentate of the 50000-cut off membrane (Fig. 3). One possible explanation of this discrepancy is the presence of different inhibitory agents produced by *S. hominis* αM. One of the agent may inhibit *Mycobacterium* spp., while the other may be responsible for the inhibition of *Micrococcus* spp.. Another reason for this difference may be the increased sensitivity to the inhibitory agent displayed by *M. luteus*. Blockage of the filter pores due to excessive amounts of proteins found in the culture supernatant as well as absorption of the inhibitory agent to the membrane may also contribute to the loss of activity (48).

This molecular weight range does not coincide with the molecular weights of known bacteriocins of gram-positive organisms. Bacteriocins produced by Staphylococci tend to have a molecular weight less than 10000 MW or greater than 50000 MW (65-68). For example, the bacteriocins AU-26, Bac1829, IYS2, D91, and the lantibiotics epidermin, gallidermin, and Pep5 have molecular weights of 2760 Da, 6418 Da, 5000 Da, 76000 Da, 2164 Da, 2164 Da, and 3488 Da, respectively (94, 66, 68, 52, 26, 50, 43). In this study, the activity of the inhibitory agent was recovered in the retentates and the filtrates, including the retentates of the membranes with higher molecular weight cutoffs, which was not expected. The activity that was detected may be due to the formation of high molecular weight aggregates of the inhibitory agent, an observation which has been made in other studies of bacteriocins of gram-positive organisms. Ammonium sulfate precipitated plantaricin S was filtered through various pore membranes (1000000, 300000, 100000, 30000, 10000, and 3000 MW) in a similar fashion (48). It was revealed that when membranes with higher molecular weight cutoff were used, amounts of

plantaricin S were recovered in both the retentate and the filtrate. They suggested that plantaricin S may exist in smaller units (2.5 kDa as determined by SDS-PAGE) and as larger aggregates. Sahl and Brandis also remarked that other staphylococcins, such as staphylococcin 1580, 414, and 462, were found as aggregates of high molecular weight and upon SDS treatment dissociate into smaller subunits. This may be the case for the inhibitory agent produced by *S. hominis* αM.

When other strains of *S. hominis* were examined for production of the anti-Mycobacterial agent, no such activity was recovered in a solid or a liquid media. Therefore, the anti-Mycobacterial activity produced by *S. hominis* αM appears to be unique amongst the *S. hominis* strains tested. However, *S. hominis* E36B-4 did produce an inhibitory activity against *S. hominis* αM as well as strains 27847 and E56A-2. This activity could be explained by the presence of another different inhibitory agent in this strain.

Production of the inhibitory agent by *S. hominis* strain αM was detected as early as five hours post-inoculation. Thus production appeared to occur during logarithmic phase (Fig. 6). This finding is similar to the production of the bacteriocins IYS2 (68), plantaricin C19 (3), and plantaricin S (48). Other bacteriocins of gram-positive bacteria are produced during the stationary phase. The bacteriocins Bac1829 (66), cerein (71), and mersacidin (18) accumulate from the onset of stationary phase while plantaricin T is produced late in stationary phase (48). After the onset of production, the inhibitory activity accumulated during stationary phase with maximum activity levels found 9 hours after inoculation. This maximum activity level was maintained at a constant level even 24 hours after inoculation, indicating that the inhibitory agent produced by *S. hominis* αM

is relatively stable in culture. The bacteriocins, Bac1829 (66) and mersacidin (18), also follow this pattern. However, this is not the case for the bacteriocin, cerein, which loses inhibitory activity three hours after the onset of production by either inactivation or degradation (71). As mentioned previously, the indicator, *M. luteus* ATCC 4698, detected production of the inhibitory agent earlier (five hours) than the indicator, *M. smegmatis* (seven hours after inoculation) perhaps because of the greater sensitivity of *M. luteus* to the inhibitory agent or that different agents are produced at different times.

It was determined that *S. hominis* αM may contain up to seven plasmids as detected by electrophoresis. Although some of the bands are comparable to the other *S. hominis* strains, for the most part, the bands are unique for *S. hominis* αM, as shown in Fig. 7. Curing was employed to determine what, if any, role does a particular plasmid has in the production and immunity process, since under certain growth conditions or upon exposure to a chemical curing agent, the rate of plasmid elimination may be increased (109). If the anti-Mycobacterial activity is plasmid associated, attempts to cure the plasmid utilizing mitomycin C, rifampicin, and ethidium bromide have so far been unsuccessful as all bacteria screened still retained this inhibitory activity. It must be taken into consideration that the curing studies involving rifampicin could not be conducted under the same conditions as described by Wannamaker (95) because of the lack of a growing media in which no detectable inhibitory activity was observed. Since the cured organisms are no longer able to produce the immunity protein for the inhibitory agent, it is imperative that no agent be present. Therefore, BHI was utilized because the smallest inhibition zones were observed when *S. hominis* αM was grown in this media as shown in Table 8. Furthermore, the cells were washed to remove any cell bound agent.

Reasons that the curing procedure may not have eliminated inhibitory activity include the following. First, the genetic sequences responsible for the production and immunity of this inhibitory agent may be located on the host organism's chromosome or on a transposon. Only the successful isolation and sequencing of the inhibitory agent will confirm this finding. If proven to be true, then this finding will be unique for Staphylococci. However, several bacteriocins produced by other gram-positive bacteria have been chromosomally located. Examples of structural genes located on the host organism's chromosome include *scnA* of the lantibiotic SA-FF22 (41), *spaA* of the lantibiotic subtilin (6), *salA* of the lantibiotic salivaricin A (83), as well as the structural genes of mersacidin (106) and sakacin 674 (108). The structural gene, *nisA*, of the lantibiotic nisin A is located on the conjugative transposon, Tn5301, which has been found in both the host organism's chromosome as well as on plasmids (15). Second, it is possible that these procedures did not successfully remove the plasmid that is responsible for the production of the agent from the host organism. Plasmid extractions performed on random isolated colonies revealed possible removal of some of the plasmids. The colonies that were examined were most likely *S. hominis* αM because the colonies were found to be gram-positive, catalase-positive and their plasmid band pattern are similar to that of *S. hominis* αM. When examined by the deferred antagonism method, these isolated colonies did not inhibit *S. hominis* αM, however, all colonies inhibited *Mycobacterium* spp. and *M. luteus* 11, which resembles the pattern exhibited by *S. hominis* αM. To positively determine whether or not the genes for the anti-Mycobacterial agent production and immunity are found on a plasmid, the plasmids of *S. hominis* αM

must be isolated and transformed into a non-producing strain. These transformed cells can be examined for the expression of the anti-Mycobacterial agent.

After a successful isolation and purification procedure is developed, other characteristics can be determined and studied regarding this inhibitory agent. The isolated agent can be sequenced and the molecular weight and the protein configuration determined, thus leading to a definitive classification for the antimicrobial agent. The inhibitory agent might be classified as a bacteriocin of gram-positive bacteria, possibly a lantibiotic (type A or B). Once the agent is purified and isolated, probes can be developed to determine the location of the structural gene that are responsible for the production of the inhibitory agent. Other characteristics such as the agent interaction or contact with the host cell, mode of action, reasons for the production of the agent and the isolation of the protein associated with host organism immunity towards the inhibitory agent are possible subjects to investigate. Most importantly, the agent can be further examined for its inhibitory activity towards pathogenic strains of *Mycobacterium* such as *M. tuberculosis* and studies can be conducted on the practicality of this agent for therapeutic usage towards this organism.

CHAPTER V

CONCLUSION

S. hominis strain αM has been shown to produce an inhibitory agent and this agent has many similarities to various bacteriocins of gram-positive bacteria, as well as anti-microbial agents produced by Staphylococcal species. Besides possessing an active biological protein moiety, the substance is heat-stable and acid-stable. As with some anti-microbial agents produced by Staphylococci, this agent is base-labile and is produced during mid-logarithmic phase. A broad spectrum of activity is observed which is indicative of bacteriocins produced by gram-positive bacteria.

Although several similarities are seen, various difference do exist which make this inhibitory agent unique. First, the spectrum of activity of this agent includes inhibition of *Mycobacterium* spp. making this activity unique for Staphylococcal species, as well as most gram-positive organisms. The apparent approximate molecular weight of 12,000-30,000 is larger than most bacteriocins of gram-positive bacteria.

Isolation and purification of the inhibitory agent produced by *S. hominis* strain αM is the next step into gaining more insight on its composition and characteristics, both of which will lead to the eventual classification of the agent. After purification, research can be conducted to see if this agent could be used as a therapeutic against pathogenic *Mycobacterium* species. Because of the increasing number of drug resistant pathogenic Mycobacteria, continued research on this particular inhibitory agent could be of great importance to the medical community.

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1989 to 1991 — **U.S. Department of Agriculture (Insect Reproduction)**
Junior Research Assistant or 1040 Student
Prepared various diets for Gypsy Moth, Tobacco Horn Worm, Silkworm. Mated insects for use in pest control research. Performed the brain dissections for pheromone extraction. Conducted preliminary field studies to evaluate native moths for possible laboratory mating.

1998 to Present — **Tidewater Community College, Thomas W. Moss, Jr. Campus**
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