

**Virulence Factors and Other Clinically Relevant
Characteristics of *Chryseobacterium* Species**

by

ESIAS RENIER VAN WYK

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**Supervisor: Dr. C.J. Hugo
Co-supervisor: Prof. P.J. Jooste**

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I declare that the dissertation hereby submitted by me for the Master of Science degree at the University of the Free State is my own independent work and has not been submitted by me at another university/faculty. I further cede copyright of the dissertation in favour of the University of the Free State.

E.R. van Wyk

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CHAPTER 1

INTRODUCTION

The genus *Chryseobacterium* was first described in 1994 (Vandamme *et al.*) as a member of the *Flavobacteriaceae* family. It consisted of six species originally described as *Flavobacterium* species namely *Flavobacterium balustinum*, *Flavobacterium gleum*, *Flavobacterium indologenes*, *Flavobacterium indoltheticum*, *Flavobacterium meningosepticum* and *Flavobacterium scophthalmum*. The reason for placing formerly known *Flavobacterium* species in another genus, was because of the advent of molecular taxonomy and a polyphasic approach to bacterial systematics (Stackebrandt and Goebel, 1994; Vandamme *et al.*, 1996).

Chryseobacterium is still a rapidly evolving genus with many new additions having been made over the last few years. Since the beginning of 2006, no less than 23 new species have been proposed, including *Chryseobacterium piscium* (de Beer *et al.*, 2006); *Chryseobacterium hispanicum* (Gallego *et al.*, 2006); *Chryseobacterium soldanellicola* and *Chryseobacterium taeanense* (Park *et al.*, 2006); *Chryseobacterium taiwanense* (Tai *et al.*, 2006); *Chryseobacterium wanjuense* (Weon *et al.*, 2006); *Chryseobacterium luteum* (Behrendt *et al.*, 2007); *Chryseobacterium haifense* (Hantsis-Zacharov and Halpern, 2007); *Chryseobacterium caeni* (Quan *et al.*, 2007); *Chryseobacterium daeguense* (Yoon *et al.*, 2007); and *Chryseobacterium flavum* (Zhou *et al.*, 2007). Currently, 36 species are included in the genus, but not all have yet been validated (Euzéby, 2008).

Several of the species included in the genus *Chryseobacterium* have been shown to be pathogenic to certain species of fish and frogs (Harrison, 1929; Olson *et al.*, 1992; Mudarris *et al.*, 1994; Mauel *et al.*, 2002). *Chryseobacterium gleum* was isolated from human clinical samples (Holmes *et al.*, 1984) and *Chryseobacterium indologenes* has also been found in wounded or ill patients (Kienzle *et al.*, 2000; Cascio *et al.*, 2005).

During the course of human disease caused by a pathogen, the pathogen will make use of a range of different enzymes to prolong the infection or to aid the pathogen in colonizing the host organism. These enzymes are commonly known as virulence factors (Madigan *et al.*, 2000). The potential for pathogenicity of an organism can be determined by testing for the presence of common virulence factors such as chondroitinase, coagulase, DNase, elastase, fibrinolysin, hyaluronidase, lecithinase, lipase and proteases (Edberg *et al.*, 1996; Pavlov *et al.*, 2004). Other methods that can also be used to give an indication of the pathogenicity of an organism, are to test the resistance to antimicrobials and resistance to disinfectants.

Since pathogenic characteristics of an organism may be regarded as negative, it was decided to also look for possible positive characteristics of the genus *Chryseobacterium*. One of the few organisms currently known to be able to degrade aflatoxin B1 is *Nocardia corynebacteroides* which was formerly included in the genus *Flavobacterium* as *Flavobacterium aurantiacum* (Alberts *et al.*, 2006). As the genus *Chryseobacterium* had its origin in the *Flavobacterium* genus, it was thought that the currently known *Chryseobacterium* species should be tested for the ability to degrade aflatoxin B1.

The aims of this study were, therefore, to

- 1) Perform a literature review to give an overview of the negative (potentially pathogenic characteristics) and possible positive characteristics (degradation of aflatoxin B1) of species in the genus *Chryseobacterium*.
- 2) Evaluate the potential of 14 of the currently known species of *Chryseobacterium* for pathogenicity by determining a range of virulence factors as well as other characteristics such as resistance to antimicrobials and resistance to commercially available disinfectants.

- 3) Screen 14 of the currently known species of the genus *Chryseobacterium* for their aflatoxin B1 degradation abilities.

CHAPTER 2

LITERATURE REVIEW

1. Introduction

The *Chryseobacterium* genus had its origin in the *Flavobacterium* genus. In the first edition of Bergey's Manual of Determinative Bacteriology the genus *Flavobacterium* was made up of 46 yellow pigmented, mainly Gram-negative, species (De Beer, 2005). However, with the passing of time, more and more of the species were moved to other genera until in 1984 only seven species remained, namely *Flavobacterium aquatile*, *F. balustinum*, *F. breve*, *F. meningosepticum*, *F. multivorum*, *F. odoratum* and *F. spiritivorum* (Vandamme *et al.*, 1994; De Beer, 2005).

In 1985 a proposal was made for a new family to accommodate *Flavobacterium*-like organisms, namely the *Flavobacteriaceae* (Jooste, 1985). This proposal was accepted by Reichenbach in 1989 and the family was placed in the Order Cytophagales. In 1992 the family *Flavobacteriaceae* was validated (Reichenbach, 1992). Many flavobacterial species that were associated with spoilage or pathogenicity have since been moved to other genera such as *Bergeyella*, *Chryseobacterium*, *Empedobacter* and others in the family *Flavobacteriaceae* (De Beer, 2005).

Many of the species of the *Chryseobacterium* genus are regarded as food spoilage organisms (Jooste and Hugo, 1999; Hugo and Jooste, 2003). Although some species in the genus have been isolated from diseased patients (*C. indologenes* and *C. gleum*) and fish (*C. scophthalmum*), the pathogenic characteristics of species in this genus are not known. Another aspect about this genus that has also not yet been clarified is whether the

species have the ability to break down or degrade clinically important toxins, e.g. the aflatoxins. In the study by Ciegler *et al.* (1966), a *Flavobacterium aurantiacum* strain, now more correctly known as *Nocardia corynebacteroides*, demonstrated aflatoxin degradation ability. Since *Chryseobacterium* had its origin in the *Flavobacterium* genus, the question arose as to whether *Chryseobacterium* species would be able to degrade aflatoxins.

The main aims of this literature review were, therefore, to give an introduction to the genus *Chryseobacterium* discussing its current taxonomic status (classification, description) and sources of isolation. A second aim was to illustrate what disease is, which factors may give an indication of an organism's pathogenic characteristics, and what role antimicrobials and disinfectants play against pathogenic bacteria. The third and last aim was to discuss the mycotoxins, especially aflatoxins, as clinically important agents in human illness and the role that microorganisms may play in its degradation.

2. *Chryseobacterium*

2.1. Classification

The genus *Chryseobacterium* was first suggested by Vandamme *et al.* (1994). It initially consisted of six species (*C. balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*) that were moved from the genus *Flavobacterium* based on an rRNA study. *Chryseobacterium gleum* was chosen as the type species for the genus.

The nomenclature remained static until a few years ago, when several new species were proposed and validated. New species are currently described almost on a monthly basis. Two species have since also been moved to a new genus *Elizabethkingia*, namely *E. meningoseptica* and *E. miricola* (Kim *et al.*, 2005b). The currently known and validated *Chryseobacterium* species are listed in Table 2.1. A noted exception is also listed, namely *C. proteolyticum* (Yamaguchi and Yokoe, 2000) which was not validly

published.

Table 2.1: Currently known species in the *Chryseobacterium* genus.

Name (year of isolation, if known)	Isolated from	Described by
<i>C. aquaticum</i> *	Water reservoir, Buyeo, Korea	Kim <i>et al.</i> , 2008
<i>C. aquifrigidense</i> *	Water-cooling system, Gwangyang, Korea	Park <i>et al.</i> , 2008
<i>C. arothri</i> *	Pufferfish kidneys, Kaneohe Bay, O‘ahu, Hawai‘i	Campbell <i>et al.</i> , 2008
<i>C. balustinum</i> (1959)	Blood of fresh water fish, France	Holmes <i>et al.</i> , 1984
<i>C. bovis</i> (2004)*	Raw cow milk, Israel	Hantsis-Zacharov <i>et al.</i> , 2008
<i>C. caeni</i> (2006)*	Bioreactor sludge,	Quan <i>et al.</i> , 2007
<i>C. daecheongense</i>	Sediment from fresh water lake, Lake Daecheong, Korea	Kim <i>et al.</i> , 2005a
<i>C. daeguense</i> (2007)*	Wastewater from textile dye works, Korea	Yoon <i>et al.</i> , 2007
<i>C. defluvii</i> (2003)	Activated sewage sludge, Germany	Kämpfer <i>et al.</i> , 2003
<i>C. flavum</i> *	Polluted soil, Jiangsu Province, China	Zhou <i>et al.</i> , 2007
<i>C. formosense</i> (2004)	Rhizosphere from garden lettuce, Taiwan	Young <i>et al.</i> , 2005
<i>C. gambrini</i> *	Beer-bottling plant, Germany	Herzog <i>et al.</i> , 2008
<i>C. gleum</i> (1979)	High vaginal swab, London, UK	Holmes <i>et al.</i> , 1984
<i>C. gregarium</i> *	Decaying plant material	Behrendt <i>et al.</i> , 2008
<i>C. haifense</i> (2004)	Raw milk, Israel	Hantsis-Zacharov and Halpern 2007
<i>C. hispanicum</i> (2003)*	Drinking water, Sevilla, Spain	Gallego <i>et al.</i> , 2006
<i>C. hominis</i> *	Hospitals, Belgium	Vanechoutte <i>et al.</i> , 2007
<i>C. indologenes</i> (1958)	Trachea at autopsy, USA	Yabuuchi <i>et al.</i> , 1983
<i>C. indoltheticum</i> (1951)	Marine mud	Campbell and Williams, 1951
<i>C. jejuense</i> *	Soil samples, Jeju, Korea	Weon <i>et al.</i> , 2008
<i>C. joostei</i> (1981)	Raw tanker milk, RSA	Hugo <i>et al.</i> , 2003
<i>C. luteum</i> *	Phyllosphere of grasses	Behrendt <i>et al.</i> , 2007

<i>C. molle</i> *	Beer-bottling plant, Germany	Herzog <i>et al.</i> , 2008
<i>C. pallidum</i> *	Beer-bottling plant, Germany	Herzog <i>et al.</i> , 2008
<i>C. piscium</i> (1996)	Freshly caught fish in South Atlantic Ocean (RSA)	De Beer <i>et al.</i> , 2006
<i>C. proteolyticum</i> †	Rice-field soil, Japan	Yamaguchi and Yokoe, 2000
<i>C. scopthalmum</i> (1987)	Gills of diseased turbot, Scotland, UK	Mudarris <i>et al.</i> , 1994
<i>C. shigense</i>	Lactic acid beverage, Japan	Shimomura <i>et al.</i> , 2005
<i>C. soldanellicola</i> (2005)	Roots of sand-dune plants, Korea	Park <i>et al.</i> , 2006
<i>C. soli</i> *	Soil samples, Jeju, Korea	Weon <i>et al.</i> , 2008
<i>C. taeanense</i> (2005)	Roots of sand-dune plants, Korea	Park <i>et al.</i> , 2006
<i>C. taichungense</i>	Contaminated soil, Taiwan	Shen <i>et al.</i> , 2005
<i>C. taiwanense</i> *	Soil isolate, Taiwan	Tai <i>et al.</i> , 2006
<i>C. ureilyticum</i> *	Beer-bottling plant, Germany	Herzog <i>et al.</i> , 2008
<i>C. vrystaatense</i>	Raw Chicken, RSA	De Beer <i>et al.</i> , 2005
<i>C. wanjuense</i> *	Greenhouse soil growing lettuce, Korea	Weon <i>et al.</i> , 2006

*: Not yet validated

†: Not validly published

2.2. Description

Chryseobacterium cells are Gram-negative rods with parallel sides and rounded ends. They do not form spores and are non-motile. The cells are typically 0.3 -0.6 µm wide and have a length of 1 to 10 µm. The entire genus is chemoorganotrophic and the metabolism is strictly aerobic with *C. scophthalmum* being the exception by also exhibiting a fermentative metabolism (Mudarris *et al.*, 1994).

The optimum growth temperature lies in the range of 25 - 35 °C. There are no intracellular granules of poly-β-hydroxybutyrate and sphingophospholipids are absent. The genus is catalase, oxidase and phosphatase positive. On solid media the growth is typically pigmented with a yellow to orange pigment of a flexirubin type (Vandamme *et al.*, 1994). The colonies are circular, convex or low convex, smooth, shiny and with entire edges. Menaquinone 6 tends to be the only or main respiratory quinone. Most of the species in the genus *Chryseobacterium* exhibit a high tolerance towards sodium chloride (NaCl) at concentrations of 0 to 2%. The DNA base composition ranges from 35 to 36 mol% guanine plus cytosine (Bernardet *et al.*, 2002).

2.3. Sources

2.3.1. Environmental Sources

Several of the *Chryseobacterium* species have been found in soil namely: *Chryseobacterium flavum* (Zhou *et al.*, 2007), *C. jejuense* (Weon *et al.*, 2008), *C. proteolyticum* (Yamaguchi and Yokoe, 2000), *C. soli* (Weon *et al.*, 2008), *C. taichungense* (Shen *et al.*, 2005), *C. taiwanense* (Tai *et al.*, 2006) and *C. wanjuense* (Weon *et al.*, 2006). Some species have also been found on or around the roots of growing plants namely: *C. formosense* (Young *et al.*, 2005), *C. luteum* (Behrendt *et al.*, 2007), *C. soldanellicola* and *C. taeansense* (Park *et al.*, 2006). *Chryseobacterium gregarium* was isolated from decaying plant matter (Behrendt *et al.*, 2008).

Other species have been found in watery environments such as marine mud (*C. indoltheticum*, Campbell and Williams, 1951), freshwater lake sediment (*C. daecheongense*, Kim *et al.*, 2005a), a water reservoir (*C. aquaticum*, Kim *et al.*, 2008), a water-cooling system (*C. aquifrigidense*, Park *et al.*, 2008) and in one case drinking water (*C. hispanicum*, Gallego *et al.*, 2006). Many of the remaining species were found on foodstuffs, such as fish (*C. arothri*, Campbell *et al.*, 2008; *C. balustinum*, Holmes *et al.*, 1984; *C. piscium*, De Beer *et al.*, 2005; and *C. scopthalmum*, Mudarris *et al.*, 1994), milk (*C. bovis*, Hantsis-Zacharov *et al.* 2008; *C. haifense*, Hantsis-Zacharov and Halpern, 2007; *C. joostei*, Hugo *et al.*, 2003), chicken (*C. vrystaatense*, De Beer *et al.*, 2005) and in a lactic acid beverage (*C. shigense*, Shimomura *et al.*, 2005). Four species has also been isolated from various beer bottling plants (*C. gambrini*, *C. molle*, *C. pallidum* and *C. ureilyticum*, Herzog *et al.*, 2008).

2.3.2. Industrial Sources

Three species have been isolated from industrial waste. *Chryseobacterium defluvii* was isolated from a wastewater treatment plant (Kämpfer *et al.*, 2003), *C. caeni* from sludge from a bioreactor (Quan *et al.*, 2007) and *C. daeguense* from wastewater from textile dye works (Yoon *et al.*, 2007). The significance of these species at these sites, are still unknown.

2.3.3. Clinical sources

Chryseobacterium gleum has been found in several human clinical samples such as high vaginal swabs, dialysis fluid, cerebrospinal fluid, wound swabs, etc. (Holmes *et al.*, 1984). *Chryseobacterium indologenes* was first found during an autopsy in a human trachea and since then has been found, several times, in burn wounds. The death of one of the patients was linked to *Chryseobacterium* bacteraemia (Kienzle *et al.*, 2000). There has also been one reported case where *C. indologenes* has caused bacteraemia in a diabetic child (Cascio *et al.*, 2005). The name *C. hominis* has been proposed for several strains isolated from clinical institutions (Vaneechoutte *et al.*, 2007).

Chryseobacterium indologenes is also a pathogen of the leopard frog (*Rana pipiens*; Olson *et al.*, 1992) and bullfrogs (*Rana castesbeiana*; Mauel *et al.*, 2002). At least two other *Chryseobacterium* species are also pathogenic towards fish. *Chryseobacterium balustinum* showed pathogenic characteristics in halibut (Harrison, 1929) and *C. scophthalmum* in turbot (Mudarris *et al.*, 1994).

3. Bacterial Pathogenicity

3.1. Disease

The bodies of higher organisms, such as humans and animals, provide highly favourable environments for the growth of microorganisms. This is due to the fact that the body is rich in nutrients and growth factors that are needed by chemoorganotrophic organisms. The body also provides relatively constant conditions with regard to factors such as pH and temperature. Differing regions and organs also supply varying conditions and give rise to selective areas where one type of organism is favoured over others. For instance, dry skin favours Gram-positive organisms, obligate aerobic organisms will flourish in the lungs and strict anaerobes in the large intestines (Madigan *et al.*, 2000).

Normally no microorganisms are found in the blood, organs, lymph or neural systems of the body except in the case of disease. Most frequently, microorganisms are found in the areas of the body that are exposed to microorganisms such as the skin, the mouth, the respiratory tract, the intestines and the genitourinary tract. Infection often starts at the mucous membranes which can be found throughout the body for example in the oesophagus, the mouth, the gastrointestinal and respiratory tracts, etc. (Madigan *et al.*, 2000).

Since the growth of microorganisms in the body is detrimental, animals have evolved a variety of defence mechanisms to either prevent or inhibit the growth of microorganisms. Ultimately the organisms that are able to circumvent these defences are the ones that cause disease and are commonly known as pathogens (Madigan *et al.*, 2000). The steps necessary for an infection to occur are summarized in Fig. 2.1.

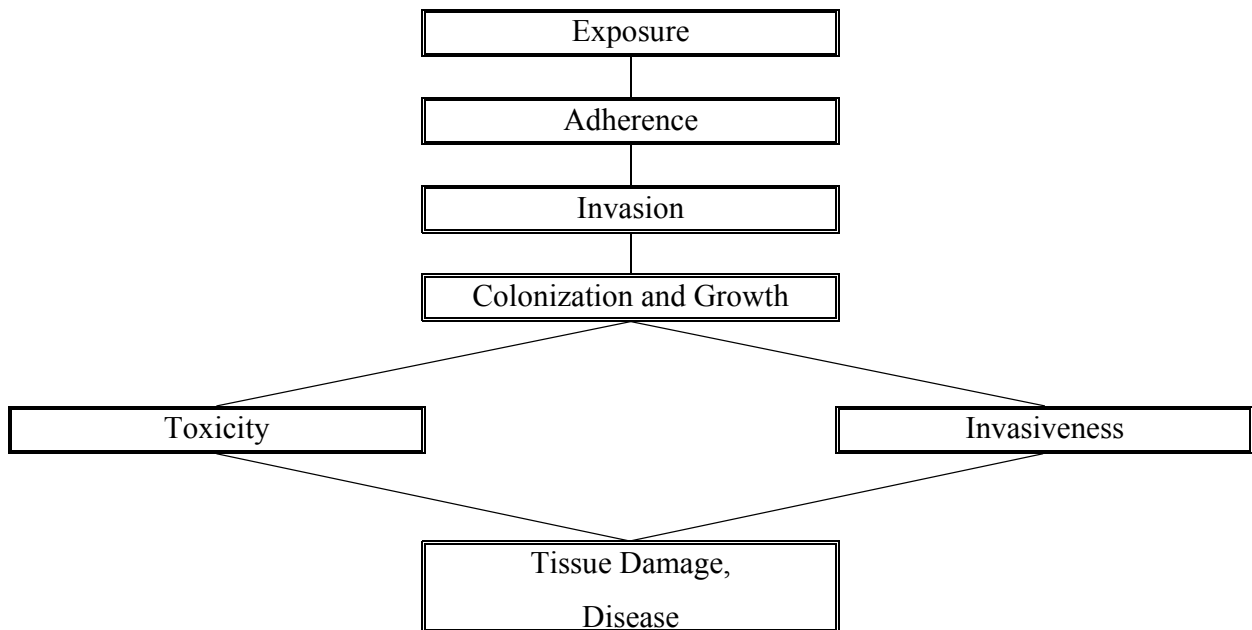


Fig 2.1 Steps of disease (Madigan *et al.*, 2000).

3.2. Entry into the Host

The ability of the organism to initiate disease in a host, is known as pathogenesis, and includes several steps such as entry into the host, colonization of the host and growth in colonized areas. The steps needed to cause disease are illustrated in Fig. 2.1 (Madigan *et al.*, 2000). First the pathogen has to gain access to the host's tissues. Most frequently this happens at the surfaces that act as microbial barriers, for example the skin and mucosal membranes.

There is a lot of evidence that suggests that pathogens, such as bacteria and viruses, can adhere specifically to epithelial cells and that there are several types of adherence. Firstly there is tissue specificity, where the pathogen only adheres to epithelial cells in a certain region. The second specificity is that of host specificity where the pathogen causes disease in one host, but cannot do so in a different host, because it binds weakly to the tissues in the one, but strongly in the other (Madigan *et al.*, 2000).

Bacteria produce macromolecules on the surface of their cells that aid in their binding to

the host's tissues. Some of these macromolecules are covalently bound to the surface of the pathogen and others are not. The non-bound molecules are usually polysaccharides that were secreted by the bacteria and which forms a polymer coat around the bacterium. If the coat is a well defined layer closely surrounding the cell it is known as a capsule. Otherwise, if the coat is a loose network of polymer fibers that extend away from the cell, it is known as a glycocalyx. If a diffuse mass of fibers is produced which seems not to be connected to any specific bacterial cell, it is called a slime layer. These coats may not only help the pathogen bind, but may also help to protect the pathogen from the host's defences (Madigan *et al.*, 2000).

3.3. Invasion

Not all pathogens need to gain access to host tissues to cause disease. A few are pathogenic because of the toxins that they synthesize, but the majority needs to penetrate the body's epithelial layers to cause disease. This process is known as invasion. The pathogen usually gains entry through small cracks or lesions in the host's skin or mucosal surfaces. After the invasion, growth usually occurs, although, some pathogens are able to initiate growth on the mucosal layers themselves (Madigan *et al.*, 2000).

3.4. Colonization and Growth

Colonization occurs after a pathogen manages to gain entry into its host and then starts to multiply. This is necessary to cause disease since the initial inoculum of the pathogen is frequently too small to cause damage. To grow, the pathogen then has to find a site which fulfills its growth requirements. These requirements include criteria such as temperature, pH and the appropriate nutrients. The host may have sufficient nutrients in its tissues, but not all nutrients are abundant or available. The nutrients may be bound in a complex form which the pathogen cannot utilize, for example glycogen. Other growth factors such as vitamins and trace elements may also be in short supply (Madigan *et al.*, 2000).

If the pathogen reaches the bloodstream, it can easily spread throughout the host and this helps it in finding a suitable location. It can also lead to a systemic infection (various growth loci) as opposed to a local infection (one location of growth). To assist the pathogen in growing successfully in the host it can produce extracellular proteins to facilitate its growth. Examples of such proteins are exotoxins and virulence factors (Madigan *et al.*, 2000).

3.5. Virulence factors of bacteria

Virulence factors are proteins that are produced by pathogens for the purpose of helping it to establish itself, to cause disease and to maintain that state. Virulence factors are frequently enzymes and are released extracellularly to aid the pathogen. Tests for virulence factors may be used to evaluate the potential of an organism to be pathogenic (Edberg *et al.*, 1996; Pavlov *et al.*, 2004). A list of some virulence factors and their activities are given in Table 2.2.

From the functions listed in Table 2.2 it can be seen that the virulence factors tend to fall into one of two broad groups. The first group consists of enzymes that help the pathogen to invade the host and move through its tissues, e.g. chondroitase which breaks down mucopolysaccharides which can be found in the mucus layers, and elastase and hyaluronidase which weaken connective tissues. The second group of enzymes is concerned with providing the pathogen with what it needs to survive, e.g. lipases which break down fats into more readily utilizable compounds and proteases which degrade proteins and may help with the acquisition of required amino acids (Madigan *et al.*, 2000).

Table 2.2: Some virulence factors of bacteria and their function.

Enzyme	Function
Chondroitase	Breaks down mucopolysaccharides (Rimler <i>et al.</i> , 1995)
Coagulase	Promotes fibrin clotting, helps protect bacteria in host tissues (Madigan <i>et al.</i> , 2000)
DNase	Breaks down DNA (Pavlov <i>et al.</i> , 2004; Seong <i>et al.</i> , 2006)
Elastase	Breaks down elastin (connective tissue; Sbarra <i>et al.</i> , 1960; Steuerwald <i>et al.</i> , 2006)
Fibrinolysin	Breaks down fibrin (Iakhiaev <i>et al.</i> , 2006)
Hyaluronidase	Breaks down hyaluronic acid (connective tissues; Madigan <i>et al.</i> , 2000)
Lecithinase	Breaks down phospholipids in cell membranes (Madigan <i>et al.</i> , 2000)
Lipase	Breaks down fats (Madigan <i>et al.</i> , 2000)
Proteases	Breaks peptide bonds of proteins (Madigan <i>et al.</i> , 2000)

3.6. Cytotoxic effects of bacterial pathogens

Many pathogens produce toxins during the course of a disease and these bacterial toxins can broadly be classified into two groups namely exo- and endotoxins.

3.6.1. Exotoxins

Toxins that are released during the growth of the pathogen are termed exotoxins. They are most commonly produced by Gram-positive bacteria and they are mostly protein molecules

while a few can be identified as specific enzymes. Usually these toxins only cause local damage where they are released, but if enough toxin is produced to enter the bloodstream, or the pathogen itself enters the bloodstream, there can be generalized toxic effects (Mims, 1986). Table 2.3 lists some of the most important exotoxins.

Some exotoxins act locally on the intestines and are known as enterotoxins. Enterotoxins are produced by microorganisms inhabiting the intestinal tract. In this case the toxins are responsible for the disease. They do not damage the intestinal cells but causes the loss of water and electrolytes into the small intestine resulting in diarrhoea (Mims, 1986).

3.6.2. Endotoxins

Endotoxins form part of the outer layer of the pathogen cell wall. During growth, small amounts of the endotoxin may be released, but mostly it will associate with the cell wall until the pathogen dies. Upon the death of the pathogen, the cell disintegrates and the endotoxin is released into the host's body. Endotoxins are less toxic than exotoxins (Mims, 1986). Endotoxins refer to phospholipid-polysaccharide-protein macromolecules associated with the cell wall of Gram-negative bacteria. The most important part of the complex is the lipopolysaccharide (LPS). The toxicity of the LPS varies between different species depending on its structure. The LPS consists of three different components: a core polysaccharide, an O-specific polysaccharide (conferring virulence and serological specificity) and a Lipid A-component which is mainly responsible for the toxicity of the molecule (Mims, 1986).

Table 2.3: Important exotoxins produced by bacteria (Mims, 1986).

Microorganism	Toxin	Action	Significance <i>in vivo</i>
<i>Clostridium perfringens</i>	α -Toxin	Phospholipase (Action on cell membrane)	Cell necrosis, haemolysis, toxaemia
<i>Clostridium tetani</i>	Toxin	Blocks action of inhibitory neurons	Overaction of motor neurons, muscle spasm, lockjaw
<i>Corynebacterium diphtheriae</i>	Toxin	Inhibits cell protein synthesis	Epithelial necrosis, heart damage, nerve paralysis
<i>Shigella dysenteriae</i>	Enterotoxin (neurotoxin)	Induces fluid loss and local cell death in intestine; vascularendothelial damage in brain	Diarrhoea, neurological disturbances
<i>Vibrio cholerae</i>	Toxin (choleraegen)	Activates adenylate cyclase and raises cAMP level in cells	Acts on intestinal epithelial cell; water and electrolyte loss into intestine
<i>Bacillus anthracis</i>	Toxic complex	Three factors form a toxic complex and cause increased vascular permeability	Oedema and haemorrhage (primary lesion); circulatory failure (systematic disease)
<i>Clostridium botulinum</i>	Toxin	Blocks release of acetylcholine	Neurotoxic signs, paralysis
<i>Staphylococcus aureus</i>	α -Haemolysin	Cytotoxic action on cell membranes	Necrosis at site of infection; systematic toxicity
	Leucocidin	Kills phagocytes	Antiphagocytic
	Enterotoxin	Action on gut nerve endings	Nausea, vomiting, diarrhea (food poisoning)
	Exfoliating toxin	Splits epidermis	Scalded skin syndrome

3.7. Antimicrobials

3.7.1. History

The discovery of antimicrobials had a profound effect on humans. The ability to control infections by microorganisms meant that humans no longer had to live in fear of plagues that could devastate entire populations. The chances for surviving surgery increased drastically as the danger of infection was lowered and diseases that were once fatal, were no longer so (Franklin and Snow, 1971).

The very first antimicrobial agent found was pyocyanase. It was discovered in Germany during the year 1888 and was produced by a bacterium then known as *Bacillus pyocyaneus* (now called *Pseudomonas aeruginosa*). It was, however, found to be toxic and unstable during human trials (Levy, 1992; McKenna, 1996).

Forty years later a major discovery was made by a scientist called Alexander Fleming when he noticed lysed colonies of bacteria on an agar plate contaminated with a *Penicillium* mould. After some study, he found and demonstrated that the mould produced a substance small enough to diffuse through agar and lyse bacteria, and named this substance penicillin (Levy, 1992; McKenna, 1996). Penicillin was, however, not used until 1940. During this period another antimicrobial group was discovered, namely the sulfonamides, which were both stable and nontoxic when used internally. Their discovery stimulated new interest in discovering antimicrobial substances. This led to the development of penicillin and the discovery of other antimicrobials (Levy, 1992).

3.7.2. Groups of Antimicrobials

Broadly speaking there are three types of antimicrobials, namely the natural antimicrobials that can be found to occur in nature (Levy, 1992), the semisynthetic antimicrobials which are natural antimicrobials which have been altered through chemical means

(Perlman, 1977; Madigan *et al.*, 2000) and finally the synthetic antimicrobials that have been manufactured *de novo* (Levy, 1992).

3.7.3. Sites of action

All antimicrobial agents have a specific site of action in the target microorganism where it will interfere with an essential life function (Lorian, 1986) or with protection mechanisms of the cell. This interference will then lead to the death of the microorganism (Lambert and O'Grady, 1992).

Antimicrobials can interfere with many functions in the cells such as the synthesis of bacterial cell wall components or the formation of the cell wall itself. Protein synthesis within the organism can be inhibited while inhibition of the synthesis of nucleic acids and membrane permeability can also prove lethal (Lambert and O'Grady, 1992). Table 2.4 shows the sites of action of antimicrobials and some of the antimicrobial compounds that target that specific site.

3.7.4. Resistance to antimicrobials

Drug resistance has been a severe limitation of chemotherapeutic substances and it has been discovered that resistance can occur in almost any organism against any substance with antimicrobial activity. The resistance to an antimicrobial agent, such as an antimicrobial, can occur in one of two ways. The first mechanism is mutation within the bacterial genome and the second is gene transfer between bacteria (Lorian, 1986).

The mutation giving rise to the antimicrobial resistance does not occur as a result of the use of antimicrobials. The use of the antimicrobials simply acts as a screening process in which the cells without any resistance immediately die off to give the bacteria that have resistance a chance to increase without as strenuous competition for resources (Lorian, 1986; McKenna, 1996).

Table 2.4: Sites of action of antimicrobial agents (Lambert and O’Grady, 1992).

Site of Action	Agent	Target
Cell Wall	Penicillins	Transpeptidase
	Cephalosporins	Transpeptidase
	Bacitracin	Isoprenylphosphate
	Fosfomycin	Pyruvyl transferase
Ribosome (Protein synthesis)	Chloramphenicol	Peptidyl transferase
	Macrolides	Translocation
	Tetracyclines	Ribosomal A site
	Aminoglycosides	Initiation complex for translation
Nucleic Acid	Quinolones	DNA gyrase (α -subunit)
	Novobiocin	DNA gyrase (β -subunit)
	Rifampicin	RNA polymerase
	Nitrofurans	DNA strands
Cell Membranes	Polymixins	Phospholipids
	Ionophores	Ion Transport

These resistance mechanisms are thus not a new development and have been around for a long time. Such resistant strains have been found in low numbers in bacterial communities that have never been exposed to antimicrobials through human medicine. These resistant strains arose because of the competition between soil bacteria for survival due to the fact that certain organisms found in soil produce antimicrobials naturally (Levy, 1992). Proof showing that antimicrobial resistance has been around for a long time, but is not widespread, was found through the examination of faecal matter of animals and Bushmen in South Africa where

resistant bacteria were found, but only in low frequencies (Levy, 1992; McKenna, 1996).

The more rapid and larger scale increase of antimicrobial resistance in recent times, has come about through the misuse and abuse of antimicrobials (Pesavento *et. al.*, 2007). Often antimicrobials are prescribed when they are not needed such as in the case of colds or flu which are caused by viruses (McKenna, 1996). People also do not complete a course of antimicrobials as prescribed, and keep the leftover tablets for later use, or ask that antimicrobials be prescribed when they are not needed (Levy, 1992). For the above reasons the research into new and more effective antimicrobials is of great importance globally since pathogens are exhibiting more and more resistance while some of the antimicrobials in use are toxic (Boudjella *et. al.*, 2006).

4. Disinfectants

Disinfection may be defined as the process of eliminating or destroying infection, which is accomplished by the use of a disinfectant (Sykes, 1972; Lombard, 1980). Antiseptic, when interpreted from its Greek origins, means ‘against putrefaction’. However, the term has been expanded upon and now includes activity against bacterial infection and sepsis. Thus by inference it now gives forth a meaning similar to that of ‘disinfectant’. There is a tendency to use the term specifically when referring to application to living tissues, most notably in surgery and hygiene (Sykes, 1972).

Disinfection is not an instantaneous occurrence, but a gradual process. This process is also influenced by the concentration of the disinfectant. A phenomenon which is commonly observed with disinfectants is the loss of lethal activity with a decrease in concentration of the disinfectant. It goes from lethal to bacteriostatic and finally ceases to have an impact on the growth of microorganisms (Lombard, 1980).

Antiseptics and disinfectants are widely used in health care organizations such as hospitals. They are used for various topical and hard surface applications. They play an essential role in the control of infection and prevention of nosocomial infections. Increased use

of antiseptics and disinfectants by the general public has also been observed. This is due to growing concerns over microbial contamination and infection risks in the general consumer and food markets (McDonnell and Russell, 1999; Fraise, 2002; Abadias *et al.*, 2008).

There is a wide variety of active ingredients to be found in different antiseptics and disinfectants. These include halogen-releasing agents, quaternary ammonium compounds, biguanides, and alcohols to name but a few (McDonnell and Russell, 1999).

4.1 Halogen-releasing agents

Disinfectants based on chlorine and iodine based compounds are the most important microbiocidal halogen compounds that are traditionally used in medical settings for disinfectant and antiseptic purposes.

4.1.1 Chlorine-releasing agents

Chlorine-releasing agents include sodium hypochlorite, chlorine dioxide and *N*-chloro compounds (for example sodium dichloroisocyanurate) (McDonnell and Russell, 1999). There is concern, although, regarding the formation of toxic byproducts (Bodik *et al.*, 2008; Murphy *et al.*, 2008; Winward *et al.*, 2008). Chlorine (Cl₂) and its derivative sodium hypochlorite (NaOCl), are still one of the most widely used disinfectants (Bodik *et al.*, 2008), despite being susceptible to inactivation by organic matter (Bodik *et al.*, 2008; Winward *et al.*, 2008).

Sodium hypochlorite containing solutions, such as household bleach, are commonly used for the disinfection of hard-surface areas. It is also capable of disinfecting spilled blood which contains HIV and HBV virus particles (McDonnell and Russell, 1999). In water, sodium hypochlorite ionizes to produce Na⁺ and the hypochlorite ion (OCl⁻), which establishes an equilibrium with hypochlorous acid (HOCl). Between pH 4 and 7, chlorine exists predominantly as HClO, whereas above pH 9, OCl⁻ predominates. The lethal activity depends on the amount of

free available chlorine in the water in the form of HClO (Abadias *et al.*, 2008). Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation, the OCl⁻ ion having a minute effect compared to undissolved HOCl (McDonnell and Russell, 1999).

Although chlorine-releasing agents are widely studied, the mode of action is still not completely known. Experimentally it has been shown that chlorine-releasing agents' bacteriocidal action is due to oxidative interaction with sulfhydryl on certain enzymes that can be found in the cell membrane (Bodik *et al.*, 2008), such as those used during oxidative phosphorylation (McDonnell and Russell, 1999). Cellular proteins' activity is also inhibited/destroyed due to the high oxidizing reactivity of chlorine (Bodik *et al.*, 2008) and evidence has also been found that chlorine-releasing agents affect bacterial DNA through the formation of chlorinated derivatives of nucleotide bases (McDonnell and Russell, 1999).

4.1.2 Iodine

Iodine, while not as reactive as chlorine, is still rapidly active and has bacteriocidal, fungicidal, tuberculocidal, virucidal and sporicidal activities. Iodine, in the form of an aqueous or alcoholic solution, has been used as an antiseptic for 150 years. Unfortunately aqueous iodine solutions are associated with staining and irritation. The aqueous iodine solutions are also unstable and several iodine species can be found in equilibrium in the solution with I₂ being responsible for the antimicrobial activity. These problems were circumvented with the development of iodophors (iodine-releasing agents). Although the antimicrobial activity was still present, these iodophores are considered to be less effective against certain fungi and spores than the aqueous iodine solutions (McDonnell and Russell, 1999).

The precise mode of action of iodine is unknown. Iodine penetrates into the microorganisms and attacks certain groups of proteins, nucleotides and fatty acids. This leads to eventual cell death (McDonnell and Russell, 1999).

4.2 Quaternary Ammonium Compounds

Quaternary ammonium compounds are an economically important class of industrial chemicals. They are mainly used as disinfectants, biocides, preservatives and detergents. However, they also have activities related to anti-electrostatics and phase transfer catalysts, which are found in fabric softeners, hair conditioners, emulsifying agents and constituents of room deodorizers and sanitizers to name a few (Kreuzinger *et al.*, 2007; Sütterlin *et al.*, 2008).

Quaternary ammonium compounds are classified as surface active agents, also known as surfactants (McDonnell and Russell, 1999; Sütterlin *et al.*, 2008). Surfactants have in their molecular structure both a water-repellant (hydrophobic) group as well as water-attracting (hydrophilic or polar) group (McDonnell and Russell, 1999). Surfactants can be classified as cationic, anionic, nonionic, and ampholytic (amphoteric) compounds depending on the charge or absence of ionization of the hydrophilic group.

In the case of quaternary ammonium compounds the molecule consists of a hydrophilic group carrying a positive charged quaternary nitrogen atom and a hydrophobic alkyl chain (Sütterlin *et al.*, 2008). Thus it is classified as a cationic agent. Cationic agents are most useful as an antiseptic or disinfectant (McDonnell and Russell, 1999).

Quaternary ammonium compounds are membrane-active agents. They target, in the case of bacteria, the cytoplasmic (inner) membrane and the plasma membrane for yeasts. The following sequence of events has been proposed after an microorganism has been exposed to a cationic agent: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. This leads to a loss of structural organization and integrity of the cytoplasmic membrane, together with other damaging effects to the cell (McDonnell and Russell, 1999).

4.3 Biguanides

4.3.1 Chlorhexidine

Chlorhexidine is probably the most widely used biocide in antiseptic products. It is found in handwashing and oral products as well as disinfectants and preservatives. This wide use is due to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Its activity is pH dependent and is greatly reduced should any organic matter be present. Chlorhexidine is a bactericidal agent. It was found that the uptake of chlorhexidine by bacteria is rapid and depended on concentration and pH. Chlorhexidine is not sporicidal and its antiviral activity is variable (McDonnell and Russell, 1999).

4.4 Alcohols

Several alcohols have been shown to be effective as antimicrobials. The alcohols most widely used as antimicrobials are ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and n-propanol. Alcohols have a rapid and broad range of antimicrobial activity. Due to a lack of sporicidal activity, alcohols are not recommended for sterilization, but are widely used in hard surface disinfection. Lower concentrations of alcohols may also be used as preservatives and to potentiate the activity of other biocides (McDonnell and Russell, 1999).

The antimicrobial activity of alcohols is less pronounced at concentrations of less than 50% and optimal at a concentration range of between 60 to 90%. The specific mode of action of alcohols is unknown but based on the increased efficacy in the presence of water it is believed that they cause damage to the cell membrane and rapid protein denaturation. Many alcohol products include low levels of other biocides (for example chlorhexidine), which remain after the alcohol has evaporated (McDonnell and Russell, 1999).

4.5 Resistance to disinfectants

Seeing as the majority of disinfectants are frequently complexes of antimicrobial agents, which inactivate more than one target in a cell, it is believed that resistance to a disinfectant is unlikely to occur as a single mutation (Karatzas *et al.*, 2007). A cause for concern is the potential for developing co-resistance to antimicrobials and disinfectants which could help in the selection of drug-resistant strains of bacteria. Lessened disinfectant susceptibility has been reported for a variety of antimicrobial resistant microorganisms (Wisplinghoff *et al.*, 2007). Reduced susceptibility to specific agents such as quaternary ammonium compounds has been known to occur. An important non-specific defence mechanism for antimicrobial resistance is membrane impermeability coupled together with efflux pumps (Karatzas *et al.*, 2007).

Efflux pumps are proteins found in the membrane. They actively transport a wide range of toxic substances, such as antimicrobials, disinfectants and dyes, out of the bacterial cell. This prevents the build up of toxic substances within the cell and in this manner helps with resistance. The wide range of substances recognized by efflux pumps has caused concern that the exposure to one substance may also lead to resistance to others (Karatzas *et al.*, 2007).

5. Toxin degradation

5.1. Mycotoxins

A wide variety of secondary metabolites are produced by fungi and many of these metabolites cause adverse effects in both humans and animals. These secondary metabolites have been named as mycotoxins. They are commonly found in the mycelium of fungi and sometimes in the spores. The negative effects of these mycotoxins, in the host, are referred to as mycotoxicoses. They are usually produced after a phase of balanced growth by the fungus. Mycotoxins are produced by a variety of fungi and can be quite diverse from each other. The production of specific mycotoxins may be limited to a few fungal species and can even be limited to a single strain (D'Mello and MacDonald, 1997).

Research into mycotoxins only started in 1960 after the death of a 100 000 turkeys after being fed contaminated groundnuts. This research led to the isolation and identification of a group of mycotoxins called aflatoxins (Bhatnagar *et al.*, 1992).

5.2. Aflatoxins

Aflatoxins are highly toxic and are mostly produced by species of the genus *Aspergillus*, most notably by the species *Aspergillus flavus* and *A. parasiticus* (Alberts *et al.*, 2006). Currently there are 18 different types of aflatoxin that have been identified with the major types being aflatoxin B₁, B₂, G₁ and G₂ (Guedes and Eriksson, 2006).

Usually the term, aflatoxin, refers to a group of difurocoumarins which is divided into two groups based on their chemical structures. The first group is the difurocoumarocyclopentenone series (B₁, B₂, B₂A, M₁, M₂, M₂A and aflatoxicol) and the second group is the difurocoumarolactone series (G₁, G₂, G₂A, GM₁, GM₂, GM₂A, and B₃).

Aflatoxins can be found on several food sources such as maize, rice, peanuts and cereals (Lötter and Kröhm, 2000) which are used in both human and animal nutrition (Sassahara *et al.*, 2005). This is an important problem since aflatoxins are highly toxic, as well as carcinogenic, towards both animals and humans. The severity of the effect depends on the dose, length of exposure, species and diet (Guedes and Eriksson, 2006). The aflatoxin series display a potency of action in the order of B₁ > M₁ > G₁ > B₂ > G₂ with aflatoxin B₁ being the most potent of the series (Sassahara *et al.*, 2005; Guedes and Eriksson, 2006).

5.2.1. Aflatoxin B₁

5.2.1.1. Carcinogenic/Mutagenic Effects

Aflatoxin B₁ is the most toxic, carcinogenic and mutagenic of the aflatoxin series (Bhatnagar *et al.*, 1992), and has been classified as a group 1 carcinogen (IARC, 1987). It is also predominantly found in cultures of *Aspergillus* as well as on food (Guedes and Eriksson, 2006). Aflatoxin B₁ inhibits a variety of cell functions when ingested such as DNA synthesis, messenger RNA synthesis, certain polymerase activities and protein synthesis. It also causes mutation by causing chromosomal aberrations, sister chromatid exchange and chromosomal strand breakages. Aflatoxins can also generate reactive oxygen species which causes lipid peroxidation, which can lead to direct cell injury or indirect damage due to the formation of malondialdehyde which is a mutagenic compound (Guedes and Eriksson, 2006). Chronic exposure to aflatoxin B₁ is dangerous and in certain parts of Africa the prevalence of aflatoxin B₁ together with the hepatitis B virus has been considered as a possible explanation for the high incidence of primary liver cancer (Alberts *et al.*, 2006).

Aflatoxin B₁, when absorbed by mammalian organisms, is converted into various derivatives by reduction and hydroxylation (Bhatnagar *et al.*, 1992). An example of these derivatives are aflatoxin M₁ (Sassahara *et al.*, 2005) and aflatoxin B₂A (Howes *et al.*, 1991).

5.2.1.2. Degradation

Many mechanisms, both physical and chemical in nature, have been tested in an attempt to detoxify aflatoxin B₁, however, none of these methods have really fulfilled criteria such as safety and cost (Mishra and Das, 2003). The focus has consequently fallen onto the development of biological detoxification methods. Unfortunately, mechanisms for the degradation of aflatoxin B₁ were found in only a few of the microorganisms that have been tested. At present, the only microorganisms that have been shown to effectively degrade aflatoxin B₁ are *Nocardia corynebacteroides* (Ciegler *et al.*, 1966) and *Mycobacterium fluoranthenvorans* (Hormisch

et al., 2004). *Corynebacterium rubrum* was shown to have a notable degradation ability (Shih and Marth, 1975; Mann and Rehm, 1977). It was also found that *Rhodococcus erythropolis* grown in liquid cultures had degradation abilities (Teniola *et al.*, 2005; Alberts *et al.*, 2006).

In the study by Ciegler *et al.* (1966), a *Flavobacterium aurantiacum* strain, now more correctly known as *Nocardia corynebacteroides*, demonstrated aflatoxin degradation ability. Since *Chryseobacterium* had its origin in the *Flavobacterium* genus, the question arose as to whether *Chryseobacterium* species would be able to degrade aflatoxins.

6. Conclusions

From the variety of different locations that species of the genus *Chryseobacterium* are found in, it is evident that human contact with this organism is a strong possibility. This is substantiated by the fact that some *Chryseobacterium* species have been found in human clinical samples e.g. *C. gleum* (Holmes *et al.*, 1984) and *C. indologenes* (Yabuuchi *et al.*, 1983), while *C. indologenes* has even been implicated as an opportunistic pathogen (Kienzle *et al.*, 2000; Cascio *et al.*, 2005). Some species are also pathogenic to animals, such as fish (*C. balustinum*, Harrison, 1929; *C. scophthalmum*, Mudarris *et al.*, 1994) and frogs (*C. indologenes*; Olson *et al.*, 1992; Mael *et al.*, 2002).

The above clinical or veterinary significance together with the fact that the genus exhibits a wide range of antimicrobial resistance (Vandamme *et al.*, 1994; Bernardet *et al.*, 2002) shows that species from the *Chryseobacterium* genus may pose a health risk should any of the species become exposed to a susceptible host. Not much is known about the pathogenic potential of the many new species that have been added to the genus and this should be investigated. Based upon possible opportunistic pathogenicity, the susceptibility towards disinfectants may also be of importance.

With regard to the aflatoxins, many degradation studies by a variety of microorganisms have been done, with very little usable results (Mishra and Das, 2003). Biological degradation of aflatoxins have been indicated in only a few organisms (Shih and Marth, 1975; Mann and Rehm, 1977; Ciegler *et al.*, 1966; Hormisch *et al.*, 2004; Teniola *et al.*, 2005; Alberts *et al.*, 2006). With so little being known about the new *Chryseobacterium* species, evaluation of their degradation potential, would add valuable information to the knowledge of the species.

CHAPTER 3

MATERIALS AND METHODS

1. Strains

The 14 *Chryseobacterium* strains used in this study are shown in Table 3.1. There are currently 36 described *Chryseobacterium* species (Euzéby, 2008). However, at the start of this study, only 14 species were available from culture collections.

All the reference strains were reactivated in Nutrient Broth (Oxoid CM67) and checked for purity on Nutrient Agar (1.5% (w/v) agar) at 25°C for 24 to 48 h. The strains were maintained in a freeze-dried state on filter paper discs and stored in screw-capped tubes at -20°C, and for longer times in glass ampoules under vacuum. Incubation temperature was 25°C unless otherwise indicated.

2. Potential Pathogenicity

To determine the potential pathogenicity of the 14 *Chryseobacterium* species, characteristics such as the haemolytic activity, enzymatic activities, antimicrobial resistance patterns and resistance to four commercially available disinfectants of these species were investigated. For determination of the haemolytic and most enzymatic activities, multi-inoculation of the media with standardized cultures were performed with a multiple-inoculation device (Jooste, 1985). The standardization entailed suspending growth from a 24 h agar slant culture in 5 ml of sterile phosphate buffer (1 N) until a density comparable to a McFarland 2 standard (Difco 0691326) had been attained.

Table 3.1: *Chryseobacterium* species used in this study.

Name	Culture Collection no.	Described by
<i>C. balustinum</i>	LMG 8329 ^T	Holmes <i>et al.</i> , 1984
<i>C. daecheongense</i>	DSM 15235 ^T	Kim <i>et al.</i> , 2005
<i>C. formosense</i>	CCUG 49271 ^T	Young <i>et al.</i> , 2005
<i>C. gleum</i>	NCTC 11432 ^T	Holmes <i>et al.</i> , 1984
<i>C. indologenes</i>	LMG 8337 ^T	Yabuuchi <i>et al.</i> , 1983
<i>C. indoltheticum</i>	ATCC 27950 ^T	Campbell and Williams, 1951
<i>C. joostei</i>	LMG 18212 ^T	Hugo <i>et al.</i> , 2003
<i>C. piscium</i>	LMG 23089	De Beer <i>et al.</i> , 2006
<i>C. scophthalmum</i>	LMG 13028 ^T	Mudarris <i>et al.</i> , 1994
<i>C. shigense</i>	DSM 17126 ^T	Shimomura <i>et al.</i> , 2005
<i>C. soldanellicola</i>	CCUG 52904 ^T	Park <i>et al.</i> , 2006
<i>C. taeanense</i>	CCUG 52900 ^T	Park <i>et al.</i> , 2006
<i>C. taichungense</i>	CCUG 50001 ^T	Shen <i>et al.</i> , 2005
<i>C. vrystaatense</i>	LMG 22846 ^T	De Beer <i>et al.</i> , 2005

2.1 Haemolytic Activity

To test for haemolytic activity, the test organisms were grown on horse and sheep blood agar plates (obtained from the Medical Microbiology Department; University of the Free State). The plates were incubated at 25 °C for 24 h. The presence of clear zones around the colonies were indicative of β -haemolysis (complete lysis of the red blood cell). Green zones around colonies indicated α -haemolysis. The greenish halo around the colony and is the result of

hemoglobin reduction to methaemoglobin in red blood cells. No haemolysis is known as γ -haemolysis (Pavlov *et al.*, 2004).

2.2 Enzymatic Activity / Virulence Factors

2.2.1 Gelatin Hydrolysis

Two methods were used for this test (MacFaddin, 1976). In Method A, 500 ml of Nutrient Broth No. 2 (Oxoid CM67) was supplemented with 5.5 g of agar (Oxoid LP0011) and boiled until the agar was completely dissolved. The medium was allowed to cool slightly before 2 g of gelatin (Merck 250 31 00 EM) was added to it. The medium was then allowed to stand for 5 min before being sterilized at 110°C for 10 min. After inoculation, the poured and set plates were incubated for 5 days before being flooded with 5 – 10 ml of Frazier's Reagent (12 g mercuric chloride + 80 ml distilled water + 16 ml concentrated HCl). Clear zones around the inoculated test organism were indicative of a positive result.

In Method B, 60 g of gelatin were added to 500 ml of distilled water and allowed to stand for 30 min. It was then heated to boiling point. Beef extract (1.5 g; Oxoid L29) and peptone (2.5 g; Oxoid L37) was then added and the mixture heated to boiling point. After allowing it to cool slightly, the pH was adjusted to 7. An amount of 10 ml of this medium was then placed into test tubes and autoclaved. The tubes were stored at -4°C until use. Stab inoculation was used and the tubes incubated at 25°C. The tubes were checked for liquefaction (gelatin hydrolysis) every 24 h for 14 days by placing them into the freezer for 2 h. If after 2 h the medium in the tube was still liquid (had not solidified), hydrolysis had occurred.

2.2.2 Chondroitinase

This method was performed according to Smith and Willette (1968), Janda and Bottone (1981), Edberg *et al.* (1996) and Pavlov *et al.* (2004). The basic medium comprised of 100 ml Heart Infusion Broth (Oxoid CM0375) and 1g of Noble Agar (Difco 214230). Aqueous solutions of respectively 4 mg/ml chondroitin sulphate A from bovine trachea (Sigma C9819-25G) and a 5% bovine albumin fraction V (Sigma A1595-50ML) were prepared. Both solutions were separately filter-sterilized using 0.20- μ m Millex-GS filter units (Millipore SLGS025OS) before mixing and adding to the molten and cooled basic medium. The poured plates were inoculated and incubated at 25°C for 48 h. The appearance of a clear zone around a colony was taken as a positive test.

2.2.3 Coagulase activity

Determination of coagulase activity was performed according to Pavlov *et al.* (2004). Rabbit coagulase plasma with EDTA (ethylenediaminetetraacetate) was used for this test. An amount of 0.5 ml of the plasma was placed into a test tube and inoculated with a single colony of the test organism from a Nutrient Agar plate, which had been incubated not more than 24 hours. The test tubes were then incubated for 24 h at 25°C. The formation of a clot in the test-tube contents was taken as a positive result.

2.2.4 DNase activity

This method was performed according to Janda and Bottone (1981), Edberg *et al.* (1996) and Pavlov *et al.* (2004). The substrate used for the DNase test was DNase Agar (Oxoid CM321) that was supplemented with 0.01% toluidine blue. After the plates were inoculated and incubated for 24 h at 25°C, the plates were flooded with 0.1% of a 1 M HCl solution. The development or appearance of either a pink halo or a zone of clearance around a colony was taken as a positive result.

2.2.5 Elastase activity

Elastase activity was performed according to Sbarra *et al.* (1960), Janda and Bottone (1981), Edberg *et al.* (1996) and Pavlov *et al.* (2004). Nutrient Agar (Oxoid CM0003) was supplemented with elastin powder from bovine neck ligament (Sigma E6527-1G) to a concentration of 1%. Plates were incubated for 48 h at 25°C and then removed and kept at room temperature for an additional 5 days. Clearing of the opaque medium around a colony was taken as a positive result.

2.2.6 Fibrinolysin activity

This method was performed according to Janda and Bottone (1981), Edberg *et al.* (1996) and Pavlov *et al.* (2004). A 100 ml amount of Nutrient Agar (Oxoid CM0003) was first autoclaved and allowed to cool to 50°C before supplementation with 280 mg of fibrinogen type III from human plasma (Sigma F4129-1G). After inoculation, incubation was at 25°C for 48 h. The formation of clear zones that were larger than 2 mm, were considered to be a positive result.

2.2.7 Hyaluronidase activity

Hyaluronidase activity was determined according to Smith and Willette (1968), Edberg *et al.* (1996) and Pavlov *et al.* (2004). The basic medium comprised of 100 ml of Heart Infusion Broth (Oxoid CM0375) and 1 g of Noble Agar (Difco 214230). An aqueous solution of 2 mg/ml of hyaluronic acid (Sigma H1504-100MG) was prepared along with a 5% bovine albumin fraction V (Sigma C9819-25G). Both solutions were separately filter-sterilized using 0.20- μ m Millex-GS filter units (Millipore SLGS025OS). After inoculation, incubation was at 25°C for 48 h. The appearance of a clear zone around a colony was taken as a positive test.

2.2.8 Lecithinase activity

Lecithinase activity was performed according to Pavlov *et al.* (2004). A 10 ml solution of 50% egg yolk enrichment (Difco 233471) was added to 90 ml of McClung Toabe agar base (Difco 294110). The agar was autoclaved separately and allowed to cool to 50°C before mixing. After inoculation, incubation was at 25°C for 72 h. The formation of a white precipitate around or beneath the inoculation spot was taken as a positive result.

2.2.9 Lipase activity

A 100 ml of Trypticase Soy Agar (BBL 211043) was supplemented with 1 ml of Tween 80 (Fluka 93781) to serve as substrate in this test. After inoculation, incubation was at 25°C for 72 h. The appearance of a turbid halo around a colony was taken as a positive result (Janda and Bottone, 1981; Edberg *et al.*, 1996; Pavlov *et al.*, 2004).

2.2.10 Proteinase activity

Skim Milk powder (Oxoid L31) was incorporated into dialysed Brain Heart Infusion Broth (Oxoid CM0375) with addition of 1.5% agar. After inoculation, the plates were incubated for 72 h at 25°C. The formation of a clear zone around the colonies was taken as a positive result (Edberg *et al.*, 1996).

2.3 Antimicrobial Resistance

The antimicrobial resistance patterns of the test organisms were determined using the Kirby-Bauer Disk Diffusion Method. The antimicrobials used are listed in Table 3.2. Bacterial suspensions were created with densities equal to the density of a MacFarland 2 standard. Using a sterile cotton swab, the above suspensions were then streaked out over an entire plate of Mueller-Hinton Agar (Oxoid CM337) in three different directions. Using a sterile forceps,

antimicrobial disks were placed onto the inoculated plates. The plates were then incubated for 48 h at 25°C. After incubation the diameters of the zones of clearance around each disk was measured and compared to Table 3.3.

Table 3.2 Antimicrobials used in this study.

Name	Dosage (µg)	Antimicrobial Group
Ampicillin	10, 25	Penicillin
Cefepime	30	Cephems
Cefotaxime	30	Cephems
Ceftazidime	30	Cephems
Ciprofloxacin	1, 5	Fluoroquinolone
Erythromycin	10, 15	Macrolide
Gatifloxacin	5	Fluoroquinolone
Gentamicin	10	Aminoglycoside
Imipenem	10	Carbapenem
Kanamycin	30	Aminoglycoside
Levofloxacin	5	Fluoroquinolone
Meropenem	10	Carbapenem
Oxacillin	1	Penicillin
Piperacillin	75, 100	Penicillin
Piperacillin Tazobactam	110	β-Lactam
Streptomycin	10, 25	Aminoglycoside

The criteria for an organism to be considered resistant, intermediate resistant or susceptible are listed in Table 3.3 which was compiled from the Performance Standards for Antimicrobial Susceptibility Testing; 17th Informational Supplement (M100-S17; Clinical and Laboratory Standards Institute, 2007).

Table 3.3 Classification of resistance or susceptibility to an antimicrobial.

	Conc. (μg)	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin	10	≤ 13	14-16	≥ 17
Cefepime	30	≤ 14	15-17	≥ 18
Cefotaxime	30	≤ 14	15-22	≥ 23
Ceftazidime	30	≤ 14	15-17	≥ 18
Ciprofloxacin	5	≤ 15	16-20	≥ 21
Erythromycin	15	≤ 13	14-22	≥ 23
Gatifloxacin	5	≤ 14	15-17	≥ 18
Gentamycin	10	≤ 13	13-14	≥ 15
Imipenem	10	≤ 13	14-15	≥ 16
Kanamycin	30	≤ 13	14-17	≥ 18
Levofloxacin	5	≤ 13	14-16	≥ 17
Meropenem	10	≤ 13	14-15	≥ 16
Oxacillin	1	≤ 10	11-12	≥ 13
Piperacillin	100	≤ 17	18-20	≥ 21
Piperacillin- Tazobactam	100/10	≤ 17	18-20	≥ 21
Streptomycin	10	≤ 11	12-14	≥ 15

2.4 Disinfectant Resistance

The four disinfectants tested against the 14 *Chryseobacterium* species, are commercially available. The disinfectants with their active ingredients are listed in Table 3.4. Disinfectants 1 and 3 are marketed for use on wounds in diluted form and disinfectants 2 and 4 for the cleaning of surfaces.

Table 3.4 The disinfectants used in this study with their active ingredients.

Disinfectant number	Active ingredient	Commercial name
Disinfectant 1	Chloroxylenol	Dettol
Disinfectant 2	Benzalkonium chloride	Sanpic
Disinfectant 3	Chlorhexidine gluconate, cetrimide	Savlon
Disinfectant 4	Poly dimethyl ammonium chloride	Virukill

The Minimum Inhibitory Concentration (MIC) method was used for determination of resistance of the test organisms to disinfectants. The protocol for this method was obtained from Prof. R. Bragg of the Department of Microbiology, Biochemistry, Biotechnology and Food Science of the University of the Free State and is summarized in Fig. 3.1.

A two fold dilution range was prepared for each disinfectant to be tested. The initial concentration prepared was 1% (1 ml of disinfectant in 100 ml of sterile dH₂O) and the concentration halved until another four concentrations of 0.5%, 0.25%, 0.125% and 0.0625% were prepared. Into each dilution was added a 100 µl of test organisms from a broth culture which was not older than 24 h. The dilutions were then left for 20 min (contact time). After 20 min, a 100 µl of each dilution was added to 5 ml of Nutrient Broth (Oxoid CM67) and incubated at 25°C for 72 h. At the same time, both a positive control (test organism) and a negative control (disinfectant) was prepared and incubated. The last tube of Nutrient Broth not to show growth was regarded as the minimum inhibitory concentration of the disinfectant for that particular test organism. However, only if the positive control showed growth, while the negative control showed no growth.

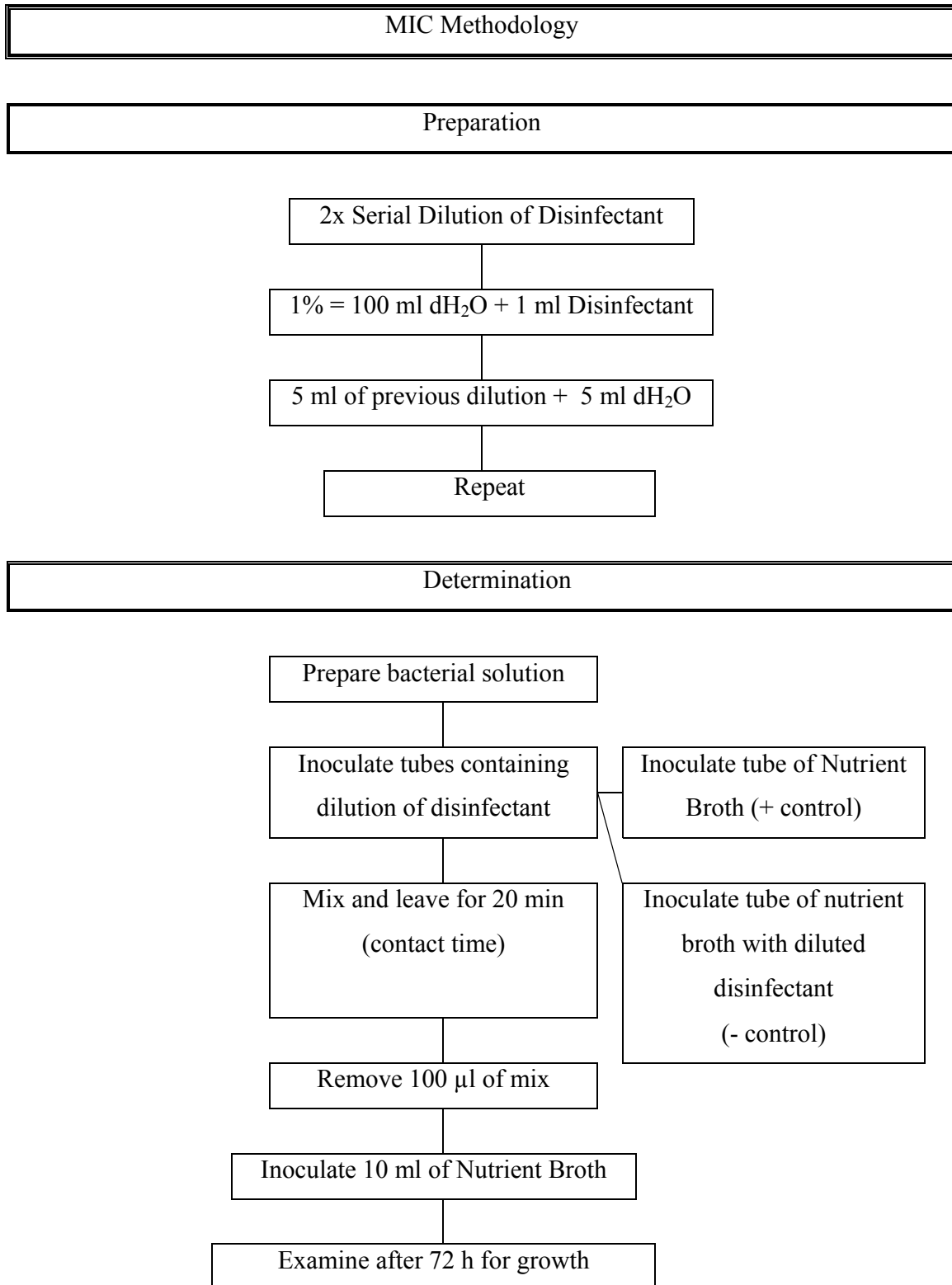


Fig. 3.1: MIC Methodology used.

3. Aflatoxin Degradation

3.1 Cultivation

The method used was according to Alberts *et al.* (2006). Each organism was streaked out on Nutrient Agar (Oxoid CM0003) to obtain single colonies. A single colony was then taken and inoculated into 10 ml of Nutrient Broth (Oxoid CM0001) and incubated at 25°C for 24 h. A 20 µl amount of this culture was then used to inoculate a flask containing 100 ml of Nutrient Broth which was then incubated for 48 h at 25°C on a shaker (100 rpm).

The cultures were then centrifuged at 10 000 rpm for 10 minutes. The extracellular fractions were aliquoted in volumes of 0.8 ml into sterile screw-cap Eppendorf tubes and supplemented with 20 µl of a 100 ppm aflatoxin B1 solution to give a final concentration of 2.4 ppm. Two controls were also prepared, a 0 h control which was immediately placed into the freezer at -20°C, and a 72 h control which was sterile Nutrient Broth with the addition of the aflatoxin B1 and which was incubated along with the extracellular samples. This was all done in triplicate. After inoculation, the samples were incubated for 72 h at 25°C on a rotary wheel, which was followed by extraction as explained in the ensuing section.

3.2 Extraction

The aflatoxin B1 was extracted from the samples using chloroform as described by Teniola *et al.* (2005) and Alberts *et al.* (2006). An amount of 0.8 ml of chloroform was added to the sample and then vortexed for 4 min. The sample was then centrifuged for 2 min at 10 000 rpm to separate the medium and the chloroform. The medium was removed and placed into a new Eppendorf tube after which the chloroform fraction was also removed and placed into a new tube and dried under nitrogen gas. This was repeated three times for each sample as well as the 72 h control sample. The samples were then placed into the -20 °C freezer to await HPLC analysis.

3.3 HPLC analysis

The samples were dissolved in 0.8 ml of methanol and then filtered (Millex-GV, Durapore, 0.22 μm) before analysis. The mobile phase that was used was a 1:1:2 ratio of v/v/v of methanol to acetonitrile to water. All of these chemicals were of HPLC grade with the water filtered to 18 M Ω in a Millipore filtration system.

The analysis was then performed using a LiChroCART 4-4 RP-C18 (5 μm) guard column (Merck) at a flow rate of 1 ml/min with the UV absorbance of the aflatoxin B1 being measured at 360 nm.

3.4 Statistical Analysis

An analysis of variance (ANOVA) was performed to determine if there were any significant differences between the samples. The Tukey-Kramer multiple comparison test with $\alpha = 0.05$ was performed (NCSS 2004).

CHAPTER 4

RESULTS AND DISCUSSION

1. Potential Pathogenicity

1.1. Haemolysis

According to Pavlov *et al.* (2004), the first step in screening organisms for potentially pathogenic characteristics, consisted of testing their ability to grow on blood agar media. The results for the haemolysis test is given in Table 4.1.

Eleven (78.6%) of the *Chryseobacterium* species tested were able to haemolyse red blood cells of either sheep or horse blood. On horse blood, 10 (71.4%) of the species exhibited α -haemolysis, while the remaining species (*C. indologenes*, *C. joostei*, *C. shigense* and *C. taichungense*) produced γ -haemolysis (no haemolysis). On sheep blood, *C. formosense*, *C. piscium* and *C. scophthalmum* exhibited α -haemolysis while *C. indologenes* was the only species that exhibited β -haemolysis. The remaining nine species exhibited no haemolysis. The species, therefore, capable of haemolysis included *C. balustinum*, *C. daecheongense*, *C. formosense*, *C. gleum*, *C. indoltheticum*, *C. piscium*, *C. scophthalmum*, *C. soldanellicola*, *C. taeanense* and *C. vrystaatense*.

1.2. Enzymatic Activity / Virulence Factors

The results for the enzymatic tests for each individual species have been listed in Table 4.2 while the percentage positive reactions of each enzymatic test produced by the 14 *Chryseobacterium* species are given in Table 4.3. Not one of the 14 *Chryseobacterium* species showed activity of the coagulase, chondroitinase, elastase and hyaluronidase enzymes. The fact that no coagulase activity was detected means that none of the species tested will be able to

Table 4.1 The haemolytic activities of the 14 *Chryseobacterium* species on horse and sheep blood.

Species	Haemolysis					
	Horse Blood			Sheep Blood		
	α	β	γ	α	β	γ
<i>C. balustinum</i>	+	-	-	-	-	+
<i>C. daecheongense</i>	+	-	-	-	-	+
<i>C. formosense</i>	+	-	-	+	-	-
<i>C. gleum</i>	+	-	-	-	-	+
<i>C. indologenes</i>	-	-	+	-	+	-
<i>C. indoltheticum</i>	+	-	-	-	-	+
<i>C. joostei</i>	-	-	+	-	-	+
<i>C. piscium</i>	+	-	-	+	-	-
<i>C. scophthalmum</i>	+	-	-	+	-	-
<i>C. shigense</i>	-	-	+	-	-	+
<i>C. soldanellicola</i>	+	-	-	-	-	+
<i>C. taeanense</i>	+	-	-	-	-	+
<i>C. taichungense</i>	-	-	+	-	-	+
<i>C. vrystaatense</i>	+	-	-	-	-	+

promote the clotting of fibrin in the bloodstream, which has been speculated as a mechanism by which pathogens protect themselves within blood (Madigan *et al.*, 2000). With no chondroitinase activity detected, which breaks down mucopolysaccharides (Rimler *et al.*, 1995) it should also lessen the danger of infection through areas that are protected by mucous layers such as can be found in the nose for example. With no hyaluronidase or elastase activity, which

breaks down connective tissues (Sbarra *et al.*, 1960; Madigan *et al.*, 2000; Steuerwald *et al.*, 2006) it should also be more difficult for any of the species tested to be able to move through tissues should an infection occur.

When the elastase test was performed, only *C. balustinum*, *C. formosense*, *C. gleum*, *C. indoltheticum*, *C. joostei*, *C. taichungens*, and *C. vrystaatense* produced very weak growth on the agar plates. It is speculated that elastin may be an inhibitor of the *Chryseobacterium* species. Further tests should, however, be performed to validate this speculation.

Table 4.2 Enzymatic activities of 14 *Chryseobacterium* species.

	Chondroitinase	Coagulase	DNase	Elastase	Fibrinolysin	Gelatinase	Hyaluronidase	Lecithinase	Lipase	Proteinase
<i>C. balustinum</i>	-	-	+	-	-	+	-	-	-	+
<i>C. daecheongense</i>	-	-	+	-	+	+	-	-	+	+
<i>C. formosense</i>	-	-	+	-	-	+	-	-	-	+
<i>C. gleum</i>	-	-	-	-	+	+	-	-	+	+
<i>C. indologenes</i>	-	-	-	-	+	+	-	+	+	+
<i>C. indoltheticum</i>	-	-	-	-	+	+	-	-	-	+
<i>C. joostei</i>	-	-	-	-	+	+	-	+	+	+
<i>C. piscium</i>	-	-	-	-	+	+	-	-	-	+
<i>C. scophthalmum</i>	-	-	-	-	+	+	-	-	-	+
<i>C. shigense</i>	-	-	+	-	+	+	-	+	+	+
<i>C. soldanellicola</i>	-	-	+	-	+	+	-	-	+	+
<i>C. taeanense</i>	-	-	+	-	+	+	-	+	+	+
<i>C. taichungense</i>	-	-	-	-	+	+	-	-	+	+
<i>C. vrystaatense</i>	-	-	+	-	+	+	-	+	-	+

Table 4.3 Percentage of positive results for enzymatic tests produced by 14 *Chryseobacterium* species.

Enzyme	Positive results out of 14	Percentage (%)
DNase	7	50
Fibrinolysin	12	85.7
Gelatinase	14	100
Lecithinase	5	35.7
Lipase	8	57.1
Proteinase	14	100

All of the 14 *Chryseobacterium* species produced proteinases and gelatinases. The proteinases and gelatinases are proteolytic enzymes that degrade proteins (breaks peptide bonds of proteins) in order to provide the organism with the required amino acids for growth (Madigan *et al.*, 2000). Gelatinase is a metalloproteinase containing zinc. It has been shown to hydrolyze substrates of gelatine and collagen and a few others (Kanemitsu *et al.*, 2001; Kayaoglu and Ørstavik, 2004; de Fátima Silva Lopes *et al.*, 2006). Proteinases have been associated with the inflammatory process, and have been shown to contribute to virulence in human and experimental animal models (Kanemitsu *et al.*, 2001).

The second most commonly produced enzyme (85.7% of the *Chryseobacterium* species produced this enzyme) was fibrinolysin, which degrades fibrin (Iakhiaev *et al.*, 2006). Only *C. balustinum* and *C. formosense* were unable to produce this enzyme. Fibrin is found in blood and plays a role in the formation of clots and scabs (Madigan *et al.*, 2000). Thus, this enzyme helps the bacteria to invade wounds on the surface of the body.

Lipases (57.1% of the *Chryseobacterium* species) and DNases (50% of the *Chryseobacterium* species) were found in about half of all the species tested. The lipases in conjunction with some of the other enzymes, breaks down cellular components (especially fats) which helps the bacterium survive in the host by freeing up components necessary for the

survival of the bacterium (Madigan *et al.*, 2000). *Chryseobacterium daecheongense*, *C. gleum*, *C. indologenes*, *C. joostei*, *C. shigense*, *C. soldanellicola*, *C. taenense* and *C. taichungense* were able to produce lipases. *Chryseobacterium balustinum*, *C. daecheongense*, *C. formosense*, *C. shigense*, *C. soldanellicola*, *C. taenense* and *C. vrystaatense* produced DNases. The DNases would free nucleic acids for DNA synthesis (Pavlov *et al.*, 2004; Seong *et al.*, 2006).

Lecithinase was found in 35.7% of the *Chryseobacterium* species. Only *C. indologenes*, *C. joostei*, *C. shigense*, *C. taenense* and *C. vrystaatense* produced lecithinases. Lecithinase breaks down phospholipids which can be found in cell membranes and as such may aid in either the destruction of the targeted cell or with cellular invasion (Madigan *et al.*, 2000).

Most of the enzymes found are known to destroy cellular components such as the DNases, gelatinases, lecithinases and the proteinases (Edberg *et al.*, 1996; Pavlov *et al.*, 2004). Others aid in the spread or invasion of the bacterium such as the fibrinolysin and the lecithinase enzymes.

In order for a bacterium to be considered pathogenic or virulent, it has to produce more than one extracellular enzyme (Edberg *et al.*, 1996). In previous clinical studies, *C. indologenes* exhibited occasional opportunistic pathogenicity to humans on several occasions (Kienzle *et al.*, 2000; Cascio *et al.*, 2005). It is also an established pathogen to certain frog species (Olson *et al.*, 1992; Mauel *et al.*, 2002). In this study, *C. indologenes* produced five enzymes (virulent factors) namely fibrinolysin, gelatinase, lecithinase, proteinase and lipase. Other *Chryseobacterium* species in this study that produced five or more enzymes were, therefore, considered possible pathogens. They include *C. daecheongense*, *C. indologenes*, *C. joostei*, *C. shigense*, *C. soldanellicola*, *C. taeanense* and *C. vrystaatense*.

According to Michel *et al.* (2005) some *Chryseobacterium* species would appear to be opportunistic pathogens. Infections caused by chryseobacteria are nosocomial in nature and are commonly seen in immuno-suppressed people (Kienzle *et al.*, 2000). Immunosuppression can come about for reasons such as chemotherapy for cancer, HIV and in the case of large scale burns to the body (Kienzle *et al.*, 2000).

1.3 Antimicrobial Resistance

Resistance of an organism to an antimicrobial, also gives an indication of the potential pathogenicity of that organism. If an organism is resistant to a specific antimicrobial, it implies that if that organism causes illness, the use of that specific antimicrobial will be ineffective. In this study the susceptibility patterns of several antimicrobials were evaluated against the 14 *Chryseobacterium* species according to the method described in Chapter 3. The results are indicated in Table 4.4. The zones of inhibition around the antimicrobial disks were measured and reported in this table along with whether the test organism can be considered susceptible or resistant to the given antimicrobial. The criteria for it to be considered resistant, intermediate or susceptible were listed in Table 3.3 in Chapter 3 and which was compiled according to the Clinical and Laboratory Standards Institute (2007).

The three species that were the most resistant to the 16 antimicrobials that were evaluated, were *C. indologenes*, *C. indoltheticum* and *C. joostei*. Each was only susceptible to five of the 16 antimicrobials tested. This implied that if these species caused illness, only a few antimicrobials will be effective in the treatment of the illness. The three species that were the most susceptible were *C. balustinum*, *C. formosense* and *C. taichungense* which had susceptibilities for between ten and twelve different antimicrobials. *Chryseobacterium daecheongense*, *C. gleum*, *C. piscium*, *C. scophthalmum*, *C. shigense*, *C. soldanellicola*, *C. taenense* and *C. vrystaatense* were susceptible to between six and nine different antimicrobials (Table 4.4).

In Table 4.5 the effectiveness of 16 antimicrobials against 14 *Chryseobacterium* species are expressed as percentage resistant, intermediately susceptible and susceptible. From this it can be seen that the cephem en quinolone classes of antimicrobials are the most effective (most of the species are susceptible) against the 14 *Chryseobacterium* species tested. The aminoglycoside class seems to be the least effective (most species were resistant) against the *Chryseobacterium* species along with the macrolide class. In the penicillin class, the species were more resistant to the older antimicrobials such as ampicillin and oxacillin and but more susceptible to the newer generation antimicrobials such as piperacillin and piperacillin-tazobactam. In the penem class,

imipenem was more effective than meropenem. If a choice would have to be made which class should be used in the treatment of an infection by any of the 14 *Chryseobacterium* species tested, it will be in the order of quinolones > cepheems > penems > penicillins.

These findings are in agreement of those of Michel *et al.* (2005). According to them a high frequency level of drug resistance can be found among aerobic gram-negative bacteria such as chryseobacteria and pseudomonads. They also stated that the antimicrobials most likely to be effective against a *Chryseobacterium* infection would be the quinolones and cephalosporins and recommended the quinolones.

The mechanisms involved with antimicrobial resistance shown by *Chryseobacterium* species are still unknown. Efflux pump systems have been found in many bacteria which have shown multiple drug resistances. These systems are responsible for the active and relatively nonspecific removal of foreign substances from the cell, which includes antimicrobial substances (Michel *et al.*, 2005).

Table 4.4 Resistance and susceptibility patterns of the 14 *Chryseobacterium* species to 16 antimicrobials.

	Conc. (µg)	<i>C. balustinum</i>	<i>C. daecheongense</i>	<i>C. formosense</i>	<i>C. gleum</i>	<i>C. indologenes</i>	<i>C. indoltheticum</i>	<i>C. joostei</i>	<i>C. piscium</i>	<i>C. scophthalmum</i>	<i>C. shigense</i>	<i>C. soldanellicola</i>	<i>C. taeanense</i>	<i>C. taichungense</i>	<i>C. vrystaatense</i>
Ampicillin	10	S	R	S	R	R	R	R	R	R	R	R	R	R	R
Cefepime	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime	30	S	I	S	R	I	I	R	I	R	R	R	I	S	R
Ceftazidime	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	5	S	S	S	S	S	S	R	S	S	R	S	S	S	I
Erythromycin	15	S	R	I	R	R	R	R	R	R	R	R	R	R	I
Gatifloxacin	5	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamycin	10	S	R	R	R	R	R	R	R	R	R	R	R	R	R
Imipenem	10	S	S	S	S	R	R	S	S	S	S	S	S	S	S
Kanamycin	30	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Levofloxacin	5	S	S	S	R	S	S	S	S	S	S	S	S	S	S
Meropenem	10	S	R	S	R	R	R	R	S	S	I	S	S	S	S
Oxacillin	1	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin	100	S	S	S	S	R	R	R	S	S	S	S	S	S	S
Piperacillin-Tazobactam	100/10	S	S	S	I	R	R	R	S	S	S	S	S	S	S
Streptomycin	10	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R = Resistant

I = Intermediate resistant

S = Susceptible

Table 4.5 The effectiveness of 16 antimicrobials against 14 *Chryseobacterium* species expressed as percentage (%) resistant, intermediately resistant and susceptible.

Antimicrobial	Class	Subclass	R (%)	I (%)	S (%)
Ampicillin	Penicillin		85.71	0	14.28
Oxacillin	Penicillin		100	0	0
Piperacillin	Penicillin		21.42	0	78.57
Piperacillin-Tazobactam	Penicillin		21.42	7.14	71.42
Cefepime	Cephems	Cephalosporin IV	0	0	100
Cefotaxime	Cephems	Cephalosporin III	42.85	35.71	28.57
Ceftazidime	Cephems	Cephalosporin III	0	0	100
Ciprofloxacin	Quinolone	Fluoroquinolone	7.14	14.28	78.57
Gatifloxacin	Quinolone	Fluoroquinolone	0	0	100
Levofloxacin	Quinolone	Fluoroquinolone	7.14	0	92.85
Imipenem	Penems	Carbapenem	14.28	0	85.71
Meropenem	Penems	Carbapenem	35.71	7.14	57.14
Gentamycin	Aminoglycoside		92.85	0	7.14
Kanamycin	Aminoglycoside		100	0	0
Streptomycin	Aminoglycoside		100	0	0
Erythromycin	Macrolide		78.57	14.28	7.14

1.4 Resistance to Disinfectants

The results of the resistance of the 14 *Chryseobacterium* species against four commercially available disinfectants are given in Table 4.6. The results are expressed as the minimum inhibitory concentration (MIC) of each disinfectant meaning that it is the lowest concentration of a disinfectant where the test organism was inhibited (no growth). Due to time constraints, testing of concentrations below 0.0625% was not possible. All positive controls showed growth (no inhibition) and the negative controls did not show any growth (inhibition).

The most effective of the four disinfectants tested was Disinfectant 4, where all of the *Chryseobacterium* species were inhibited at the lowest MIC of 0.0625% (Table 4.6). This implies that the *Chryseobacterium* species were the least resistant to this disinfectant. The active ingredient of Disinfectant 4 was poly dimethyl ammonium chloride.

The second most effective disinfectant was Disinfectant 2 with benzalkonium chloride as an active ingredient. *Chryseobacterium daecheongense*, *C. gleum*, *C. indologenes*, *C. shigense* and *C. vrystaatense*, were moderately resistant with MIC's between 0.25% and 0.125%. *Chryseobacterium joostei*, however, was the most resistant with a MIC of 0.5%. The remaining eight *Chryseobacterium* species were not resistant to this disinfectant (MIC's \leq 0.0625%) (Table 4.6).

Disinfectant 1 with chloroxylonol as an active ingredient was deemed the third most effective of the tested disinfectants. Nine of the *Chryseobacterium* species were moderately resistant with MIC's of 0.25% (*C. daecheongense*, *C. gleum*, *C. indologenes*, *C. joostei*, *C. scophthalmum*, *C. shigense*, *C. soldanellicola*, *C. taichungense* and *C. vrystaatense*). The rest of the species had MIC's of 0.125% (Table 4.6).

Table 4.6 Minimum inhibitory concentrations (MIC's) of the four disinfectants against the 14 *Chryseobacterium* species.

Species	MIC			
	Disinfectant 1	Disinfectant 2	Disinfectant 3	Disinfectant 4
<i>C. balustinum</i>	0.125 %	≤ 0.0625%	0.25%	≤ 0.0625%
<i>C. daecheongense</i>	0.25%	0.25%	0.25%	≤ 0.0625%
<i>C. formosense</i>	0.125 %	≤ 0.0625%	0.125 %	≤ 0.0625%
<i>C. gleum</i>	0.25%	0.25%	1%	≤ 0.0625%
<i>C. indologenes</i>	0.25%	0.125 %	1%	≤ 0.0625%
<i>C. indoltheticum</i>	0.125 %	≤ 0.0625%	≤ 0.0625%	≤ 0.0625%
<i>C. joostei</i>	0.25%	0.5%	0.5%	≤ 0.0625%
<i>C. piscium</i>	0.125 %	≤ 0.0625%	0.5%	≤ 0.0625%
<i>C. scopthalmum</i>	0.25%	≤ 0.0625%	0.5%	≤ 0.0625%
<i>C. shigense</i>	0.25%	0.125 %	0.25%	≤ 0.0625%
<i>C. soldanellicola</i>	0.25%	≤ 0.0625%	0.125 %	≤ 0.0625%
<i>C. taeanense</i>	0.125 %	≤ 0.0625%	0.125 %	≤ 0.0625%
<i>C. taichungense</i>	0.25%	≤ 0.0625%	0.125 %	≤ 0.0625%
<i>C. vrystaatense</i>	0.25%	0.125 %	0.5%	≤ 0.0625%

Disinfectant 3 (chlorhexidine gluconate and cetrimide as active ingredients) was deemed the least effective of the tested disinfectants. *Chryseobacterium gleum* and *C. indologenes* were the most resistant species with MIC's of 1%. *Chryseobacterium joostei*, *C. piscium*, *C. scopthalmum* and *C. vrystaatense* were also more resistant than the other species with MIC's of 0.5%. The rest of the species, except for *C. indoltheticum* were moderately resistant with MIC's ranging between 0.25% and 0.125%. *Chryseobacterium indoltheticum* was the least resistant with a MIC of ≤ 0.0625% (Table 4.6).

From these results it seems as if the *Chryseobacterium* species were more resistant to the disinfectants used on wounds (Disinfectants 1 and 3) than to the surface disinfectants (Disinfectants 2 and 4). This is unfortunate since especially *C. indologenes* and *C. gleum* have been previously associated with wound infections (Kienzle *et al.*, 2000; Cascio *et al.*, 2005).

2. Aflatoxin Degradation

The average HPLC results and standards are listed in Table 4.7 and presented in graphic form in Fig. 4.1. From these results it at first seemed as if certain species were able to degrade the aflatoxin B1. However, when the results underwent statistical analysis (ANOVA; NCSS 2004 software) no significant differences were found. The normality of the data was tested and it was found that none of the decisions could be rejected. The normality was plotted and shown in Fig 4.2. The decrease in aflatoxin witnessed should then be attributed to other circumstances than the presence of the extracellular extracts from the *Chryseobacterium* species. This could include insufficient extraction or possible degradation through photolysis.

Table 4.7 Aflatoxin B1 HPLC standards and average recovery by *Chryseobacterium* species.

Species	Standards ^a			% Standard Deviation
	0h	72h	Average % Recovery ^b	
	110376.83	90745	82.21	
Average Area	Standard Deviation	Average % Recovery ^b		
<i>C. balustinum</i>	96258.33	20691.78	87.20	18.74
<i>C. daecheongense</i>	64410.33	10782.99	58.35	9.76
<i>C. formosense</i>	87615	12325.42	79.37	11.16
<i>C. gleum</i>	69460	11435.97	62.92	10.36
<i>C. indologenes</i>	86796.66	7823.73	78.63	7.08
<i>C. indoltheticum</i>	70993.66	16907.98	64.31	15.31
<i>C. joostei</i>	103098	19061.75	93.40	17.26
<i>C. piscium</i>	69529	26015.85	62.99	23.57
<i>C. scophthalmum</i>	69600	9766.31	63.05	8.84
<i>C. shigense</i>	104442.75	42375.10	94.62	38.39
<i>C. soldanellicola</i>	95096.5	1434.71	86.15	1.29
<i>C. taeanense</i>	70995	9990.01	64.32	9.05
<i>C. taichungense</i>	107240	8419.51	97.15	7.62
<i>C. vrystaatense</i>	110100.33	11453.07	99.74	10.37

^a 0h standard: Aflatoxin from inoculation at 0h; 72h standard: Aflatoxin recovered after 72h incubation

^b Percentage Aflatoxin recovered ($[\text{Average Area} / \text{0h standard}] \times 100$)

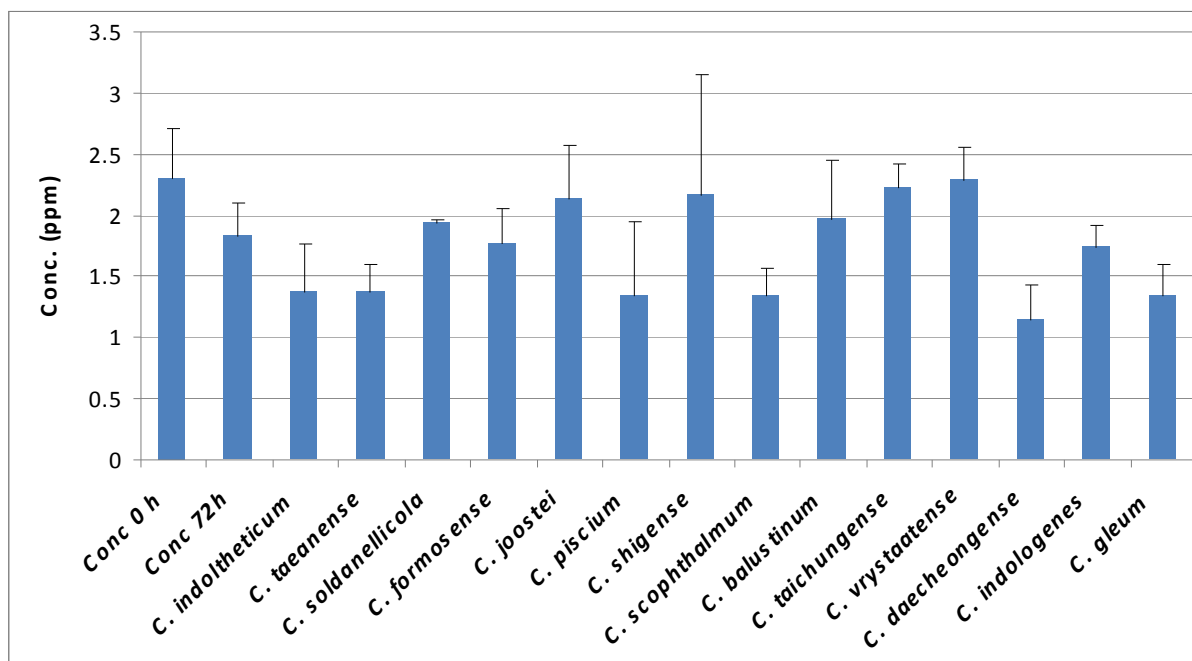


Fig. 4.1 Aflatoxin B1 concentration (in ppm) recovered from the 14 *Chryseobacterium* species.

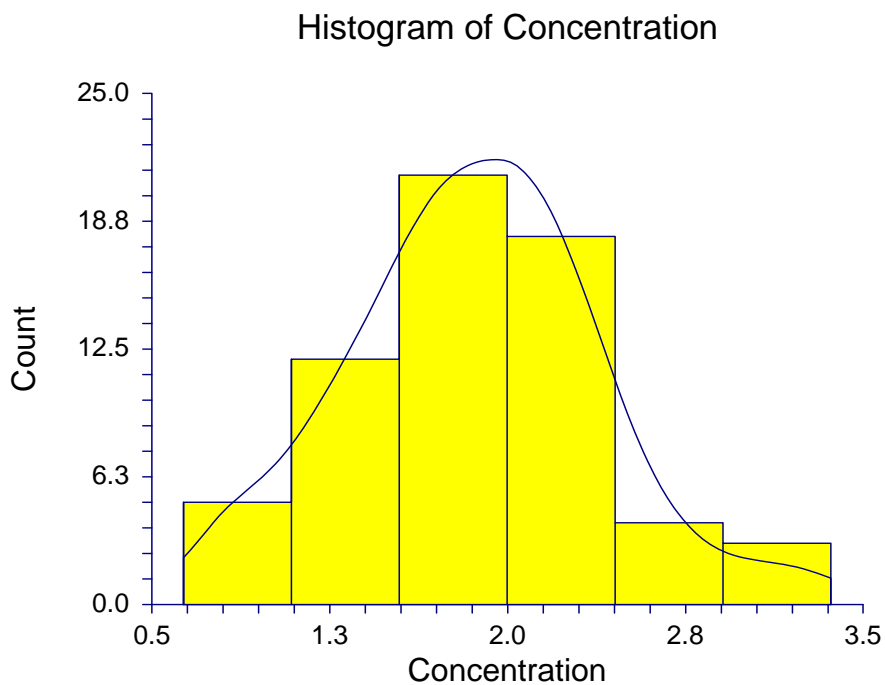


Fig 4.2 The plot of the normality test for the statistical analysis of the Aflatoxin B1 degradation by the tested *Chryseobacterium* species.

CHAPTER 5

CONCLUSIONS

The genus *Chryseobacterium* is a rapidly evolving genus with many new additions having been made over the last few years. Since the beginning of 2006, no less than 23 new species have been proposed which brings the total amount of species to date to 36. These organisms are widely distributed in the environment (soil, water, food, etc.) as well as in the clinical environment. Although some are regarded food spoilers and some as opportunistic pathogens, it is not known what the pathogenicity of the newer species are. It is also not known whether these organisms have positive characteristics such as the break down of toxins, especially aflatoxin.

The first aim of this study was to investigate the potential pathogenicity of the species in the *Chryseobacterium* genus. Although pathogenicity has been documented for *C. indologenes* and *C. gleum*, few, if any literature are available for the other species in this genus. The methods used for determination of potential pathogenicity of an organism, includes haemolysis of blood, production of specific enzymes or virulence factors, resistance to antimicrobials and resistance to detergents.

Whether an organism can haemolyse blood, is the first step in determining the potential pathogenicity of an organism (Pavlov *et al.*, 2004). In this study it was found that 11 of the *Chryseobacterium* species were able to haemolyse mostly horse blood. They were (the type of haemolysis is indicated in parenthesis) *C. balustinum* (α), *C. daecheongense* (α), *C. formosense* (α), *C. gleum* (α), *C. indologenes* (β - on sheep blood), *C. indoltheticum* (α), *C. piscium* (α), *C. scophthalmum* (α), *C. soldanellicola* (α), *C. taeanense* (α) and *C. vrystaatense* (α).

The determination of specific enzyme activities (virulence factors) of the species were used as a second step in determining the potential pathogenicity of the 14

Chryseobacterium species. The enzymes investigated (the percentage of *Chryseobacterium* species that produced the enzyme, are indicated in parenthesis) were coagulase (0%), chondroitinase (0%), elastase (0%), hyaluronidase (0%), elastase (0%), DNase (50%), fibrinolysin (85.7%), gelatinase (100%), lecithinase (35.7%), lipase (57.1%) and proteinase (100%). *Chryseobacterium indologenes* (a proven opportunistic pathogen from previous studies) and which produced five of these enzymes, was taken as the standard. The other species, therefore, considered to be potentially pathogenic are *C. daecheongense*, *C. joostei*, *C. shigense*, *C. soldanellicola*, *C. taeaanense* and *C. vrystaatense*.

The third step in the determination of potential pathogenicity was to test the resistance of the *Chryseobacterium* species to antimicrobials. The most resistant of the species tested were *C. indologenes*, *C. indoltheticum* and *C. joostei*. The most susceptible of the species was *C. balustinum*. Based upon the antimicrobial test results it would appear to be best to treat an infection of *Chryseobacterium* with antimicrobials of the quinolone class or cephem class. The penicillin class would seem to be the least effective.

The last method that was used in this study to determine the potential pathogenicity of the *Chryseobacterium* species, was to test their resistance to four commercially available disinfectants. The species with the highest resistance were *C. gleum*, *C. indologenes* and *C. joostei*. From the results it seemed if the *Chryseobacterium* species were more resistant to the disinfectants used on wounds (Disinfectants 1 and 3 with chloroxylenol and chlorhexidine gluconate, cetrimide as active ingredients, respectively) than to the surface disinfectants (Disinfectants 2 and 4 with benzalkonium chloride and poly dimethyl ammonium chloride as active ingredients, respectively). This is unfortunate since especially *C. indologenes* and *C. gleum* have been previously associated with wound infections (Kienzle *et al.*, 2000; Cascio *et al.*, 2005).

When evaluating the results of all the pathogenicity tests in this study (blood haemolysis, enzymatic activities, resistance to antimicrobials and resistance to

disinfectants), it would seem that the tested species having the highest risk as opportunistic pathogens would be *C. gleum*, *C. joostei* and *C. indologenes*. *Chryseobacterium joostei* may actually be less of a threat as it was first isolated from raw milk, which had not yet undergone pasteurization and as such, is less likely to come into contact with the general population. It would seem that *C. gleum* and *C. indologenes*, however, come into contact with people more often as they have been found in or on ill or wounded individuals on several different occasions.

The second aim of this study was to investigate a possible beneficial characteristic of the *Chryseobacterium* species by determining possible degradation of Aflatoxin B1. The initial results seemed promising where some species showed degradation of the aflatoxin. After statistical analysis of the data, no significant decrease of the aflatoxin was proven even though there was much variance within the results. The variance may have been due to exposure to light, insufficient extraction or some other reason.

In this study the knowledge on the significance of some 14 *Chryseobacterium* species were broadened. With so little known about the species of this genus and the rapidly expanding nature thereof, future research may yet lead to significant and/or beneficial findings.

CHAPTER 6

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CHAPTER 7

SUMMARY

The genus *Chryseobacterium* was first proposed in 1994 and consisted of six species, it has since rapidly expanded and currently consists of 36 different species. Most of these species have been proposed within the last 3 years. Bacteria belonging to the genus *Chryseobacterium* are aerobic, non-sporulating, non-motile, Gram-negative rods which produce a yellow pigment. *Chryseobacterium* species have been found in a variety of environmental, industrial and clinical surroundings. Several species have already exhibited pathogenic characteristics for different animals and for humans as well.

The first aim of this study was to evaluate the potential pathogenesis of 14 *Chryseobacterium* species by evaluating a variety of known virulence factors (enzymes), to evaluate their antimicrobial resistance patterns as well as to determine their resistance to four commercially available disinfectants. When the production of specific enzymes by the 14 *Chryseobacterium* species were evaluated, it was found that *C. daecheongense*, *C. joostei*, *C. shigense*, *C. soldanellicola*, *C. taeaanense*, *C. vrystaatense* together with *C. indologenes* which was regarded the standard organism, can be regarded potentially pathogenic. The species most resistant to all of the antimicrobials evaluated, were *C. indologenes*, *C. indoltheticum* and *C. joostei*. The most susceptible to the antimicrobials was *C. balustinum*. It was also found that it would be best to treat an infection of *Chryseobacterium* with antimicrobials of the quinolone class or cephem class. The penicillin class would seem to be the least effective. The disinfectant resistance was determined by determining the MIC for each tested disinfectant, with a higher MIC exhibiting more resistance. The species with the highest resistance were *C. gleum*, *C. indologenes* and *C. joostei*. The disinfectants tested were for use either on hard surfaces or on wounds (personal use). From the results obtained disinfectants for use on hard surfaces are more effective than those meant for personal use. In conclusion it can be said that *C. gleum*, *C. indologenes* and *C. joostei* may be regarded as the most

pathogenic of the 14 *Chryseobacterium* species evaluated. *Chryseobacterium joostei* may be less of a threat as it was first isolated from raw milk, which had not yet undergone pasteurization and as such, is less likely to come into contact with the general population. It would seem that *C. gleum* and *C. indologenes* however come into contact with people more often as they have been found in or on ill or wounded individuals on several different occasions.

The second aim of the study was to evaluate possible degradation of aflatoxin B1 by the 14 *Chryseobacterium* species. After statistical analysis, it was concluded that no *Chryseobacterium* species was able to degrade aflatoxin. This study, however contributed to the knowledge on the significance of these bacteria.

Keywords: *Chryseobacterium*, virulence factors, disinfectant resistance, antimicrobial resistance, Aflatoxin B1, degradation, potential pathogenicity.

HOOFSTUK 7

OPSOMMING

Die genus *Chryseobacterium* was die eerste keer voorgestel in 1994 en het bestaan uit ses spesies, sedertdien het dit vinnig uitgebrei en bestaan tans uit 36 verskillende spesies. Meeste van hierdie spesies was voorgestel binne die laaste drie jaar. Bakteriëe wat aan die genus *Chryseobacterium* behoort is aerobies, nie-sporulerende, nie-bewegende, Gram-negatiewe stawe wat 'n geel pigment produseer. *Chryseobacterium* spesies is al gevind in 'n verskeidenheid natuurlike, industriële en kliniese omgewings. 'n Paar spesies het alreeds patogeniese eienskappe getoon vir verskillende diere asook vir mense.

Die eerste doelstelling van hierdie studie was om die patogeniese potensiaal te evalueer van 14 verskillende *Chryseobacterium* spesies deur middel van die evaluasie van 'n verskeidenheid van virulensie faktore (ensieme), om die antimikrobiese weerstand patrone te evalueer, asook hul weerstand teen vier kommersiële beskikbare ontsmettingsmiddels te evalueer. Toe die produksie van spesifieke ensieme deur die 14 *Chryseobacterium* spesies ge-evalueer was, was dit gevind dat *C. daecheongense*, *C. joostei*, *C. shigense*, *C. soldanellicola*, *C. taeanense*, *C. vrystaatense* saam met *C. indologenes*, wat beskou was as die standaard organisme, beskou kon word as potensiële patogene. Die spesies wat mees weerstandbiedend was teen die ge-evalueerde antimikrobiese middels was *C. indologenes*, *C. indoltheticum* en *C. joostei*. Die mees vatbare teen die middels was *C. balustinum*. Dit was ook gevind dat dit die beste sou wees om 'n infeksie van *Chryseobacterium* te behandel met antimikrobiese middels van die 'quinolone' of 'cephem' klas. Die penisillien klas blyk om die minste effektief te wees. Die ontsmettingsmiddel weerstand was bepaal deur die minimum inhiberende konsentrasie (MIK) vir elke middel te bepaal. 'n Hoër MIK dui op hoër weerstandbiedendheid. Die spesies met die hoogste weerstand was *C. gleum*, *C. indologenes* en *C. joostei*. Die ge-evalueerde ontsmettingsmiddels was vir die gebruik

op harde oppervlaktes of op wonde (persoonlike gebruik). Vanaf die resultate verkry, blyk dit asof ontsmettingsmiddels vir gebruik op harde oppervlaktes meer effektief is as die vir gebruik op wonde. In afsluiting kan gesê word dat *C. gleum*, *C. indologenes* en *C. joostei* beskou kan word as die mees patogeniese van die 14 spesies wat ge-evalueer was. *Chryseobacterium joostei* mag dalk 'n kleiner bedreiging wees, synde dit die eerste keer in ongepasteuriseerde melk geïsoleer was en dus minder waarskynlik in kontak sal kom met die algemene publiek wat gepasteuriseerde melk drink. Daarenteen blyk dit dat *C. gleum* en *C. indologenes* meer gereeld in kontak kom met mense aangesien hulle in verskeie studies reeds gevind is in of op siek of gewonde mense.

Die tweede doelstelling van hierdie studie was om die moontlike afbraak van aflatoksien B1 by die 14 *Chryseobacterium* spesies te ondersoek. Na statistiese analise is dit bevind dat geeneen van die *Chryseobacterium* spesies in staat was om aflatoksien af te breek nie. Hierdie studie het wel bygedra tot die kennis van die belangrikheid van hierdie bacteria.

Sleutelwoorde: *Chryseobacterium*, virulensie faktore, ontsmettingsmiddel weerstand, antimikrobiese weerstand, aflatoksien B1, afbreek, potensiële patogenisiteit.