CHAPTER ONE

INTRODUCTION

1.1 Introduction

Microbes have evolved several mechanisms for iron acquisition because iron is of major importance to the process of growth and infection. Iron acquisition proteins of microbes include siderophores, hemolysins, transferrin- and lactoferrin-binding proteins and also heme- and hemoglobin-binding proteins (Woolridge and Williams, 1993).

Both *Aeromonas hydrophila* and *Vibrio cholerae* are waterborne, Gram negative bacteria classified under the Vibrionaceae family. *A. hydrophila* can cause gastroenteritis in humans (Lautrop, 1961; Sanyal *et al.*, 1975; Gracey *et al.*, 1982; Agger *et al.*, 1985) and bacteremia and septicemia in immunocompromised patients (Trust and Chapman, 1979; Harris *et al.*, 1985). *V. cholerae* is a well known pathogen which is also the causative agent of cholera, a severe debilitating diarrheal disease, often fatal if left untreated (Finkelstein, 1973; Wachsmuth *et al.*, 1994).

A. hydrophila produce several virulence factors such as enterotoxins, proteases, hemolysins and cytotoxins which have been implicated in the infection process (Cahill, 1990). In *V. cholerae*, the cholera enterotoxin and toxin-coregulated pili (TCP) has been shown to be necessary factors in its pathogenicity (Waldor and

Mekalanos, 1996). The role of other enzymes and proteins in the virulence of *V. cholerae* remains to be established.

Siderophores are low molecular mass proteins with a high affinity for ferric iron synthesized by many bacteria. Siderophore production is one of the main iron acquisition mechanisms of pathogenic bacteria (Woolridge and Williams, 1993). *A. hydrophila* produce two types of catecholate siderophores, either amonabactin or enterobactin, but not both (Barghouthi *et al.*, 1989; Zwyno *et al.*, 1992) Amonabactin which is more prevalent in *A. hydrophila* strains, has been demonstrated to acquire iron from the Fe-transferrin of vertebrate serum (Massad *et al.*, 1991). Hence it has been considered a virulence factor. Amonabactin producing strains have been noted for their resistance to lysis by complement (Massad *et al.*, 1991). *V. cholerae* also synthesize a catecholate siderophore, vibriobactin (Sigel and Finkelstein, 1978; Griffiths *et al.*, 1984), whose role in virulence still remains contoversial. A report by Sigel and Payne (1982) indicates that vibriobactin is not needed for virulence in *V. cholerae*.

Hemolysins are proteins that produce their toxic effect by inserting into the bilipid layer and disrupting the cell membrane (Howard and Buckley, 1985; Ikigai *et al.*, 1996; Song *et al.*, 1996; Menzl *et al.*, 1996). *A. hydrophila* produces two known types of hemolysins, α-hemolysin and β-hemolysin (also known as 'aerolysin') (Ljungh *et al.*, 1981; Ljungh and Wadstrom, 1982; Ljungh and Wadstrom, 1983). α-hemolysin is sensitive to proteolytic enzymes and produces incomplete hemolysis on blood agar plates, while β-hemolysin is resistant to pronase and trypsin and exhibit complete

hemolysis on blood agar plates. The aerolysin was described as an extracellular, soluble, hydrophobic protein possessing hemolytic and cytolytic activities (Berheimer and Avigad, 1974). Asao *et al.* (1984) found that the β-hemolysin purified from *A. hydrophila* possessed cytotoxic, enterotoxic and hemolytic activities. The addition of iron to the culture media drastically reduced the hemolysin production of *A. hydrophila* (Riddle *et al.*, 1981). This observation suggests that hemolysins of *A. hydrophila* may play a role in iron acquisition by lysing erythrocytes and other cells which are rich in iron content. A putative role in iron acquisition for aerolysin of *Aeromonas sobria* has also been proposed (Goebel *et al.*, 1988).

Stoebner and Payne (1988) have reported that *V. cholerae* El Tor strains secrete increased levels of hemolysin in response to iron starvation. The hemolytic activity of these strains was also seems to be regulated by *fur*, the regulatory gene involved in siderophore biosynthesis. Since *V. cholerae* has been reported to utilize heme and hemoglobin as potential sources of iron (Stoebner and Payne, 1988), this possibility is not entirely out of consideration. Similar role in iron acquisition has also been proposed for the α-hemolysin of *Escherichia coli* (Valvano *et al.*, 1986).

Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. Proteases are present in all living organisms including microbes. Microbial proteases are predominantly extracellular. Most of the *A. hydrophila* strains secrete a heat-stable metalloprotease and also a heat-labile serine protease (Leung and Stevenson, 1988a). The metalloprotease was shown to be lethal for fish (Leung and Stevenson, 1988b). The hemagglutinin(HA)/protease of *V. cholerae* O1 was shown to

be able to cleave mucin, fibronectin, lactoferrin and also cholera toxin (Finkelstein *et al.*, 1983). This protease was also found to be similar to the *P. aeruginosa* elastase (Hase and Finkelstein, 1990) and considered to aid the detachment of the bacterial cells from the intestinal cells (Finkelstein *et al.*, 1992).

Riddle *et al.* (1981) found that the presence of additional iron in the growth media greatly reduced the protease production in *A. hydrophila*. However, no reports are available on the effect of iron on the protease production in *V. cholerae*. One report (Nishina *et al.*, 1992) suggests that in *Vibrio vulnificus*, protease may play an indirect role in acquiring iron from heme compounds.

It has been shown that the environmental temperature influences the expression of virulence factors including hemolysins and proteases in *A. hydrophila* (O'Reilly and Day, 1983; Ho *et al.*, 1990; Schubert and Matzinou, 1990; Mateos *et al.*, 1993). But the study does not provide any data on the effect of growth temperature on siderophore production in the organism. Similarly, there are no reports available about the effect of growth temperature on the production of siderophores, hemolysins and proteases in *V. cholerae*.

1.2 Experimental procedure

Early detection methods for siderophores involved chemical assays specific for hydroxamates and catechols, the major known classes of siderophores (Arnow, 1937; Csaky, 1948; Evans, 1947). The development of the chrome azurol S assay for

siderophores (Schwyn and Neilands, 1987), used in this study, enables the quantification of siderophores regardless of their physico-chemical structure. The semi-quantitative microtiter plate assay (Asao *et al.*, 1986) was used to analyze the hemolysin production of the isolates. A quantitative hemolysin assay (Asao *et al.*, 1984) was also done to confirm the hemolysin production of selected *A. hydrophila* isolates. The azocasein assay method of Allan and Stevenson (1981) was employed to measure the protease activity of the isolates. Plasmid curing of the *A. hydrophila* isolates was done according to the method of Winkler *et al.* (1976), using acridine-orange as the curing agent. This technique was found to be suitable for the purpose.

1.3 Objectives of this study

Several toxins, enzymes and proteins of bacteria have been shown to play a role in the iron-acquisition process or produced in response to iron-starvation (Mekalanos, 1992). Although *A. hydrophila* and *V. cholerae* are well known pathogens of humans, the effect of iron concentrations on the production of virulence-associated factors of these species is poorly understood. In view of this, the *A. hydrophila* and *V. cholerae* isolates were studied for their ability to produce these virulence-associated factors under iron-deficient conditions.

In the present study, the effect of iron-deficiency and growth temperature on the total production of siderophores, hemolysins and proteases in clinical and environmental isolates of *A. hydrophila* was investigated. A comparison between the clinical and environmental isolates in their ability to produce these enzymes/proteins

was also done. In view of a report by Borrego *et al.* (1991) who found that 60% of the *A. hydrophila* isolates tested lost their siderophore activity after plasmid curing, the relationship between plasmid curing and siderophore production of the *A. hydrophila* isolates was also examined. Similarly, the effect of iron-limitation and growth temperature on the production of siderophores, hemolysins and proteases of *V. cholerae* was also investigated.