CHAPTER FIVE

DISCUSSION

5.1 Siderophore production in *A. hydrophila*

Siderophore production at both 28°C or 37°C increased dramatically in the iron-limiting conditions. More than 60% of the environmental isolates and 80% of the clinical isolates tested produced high levels of siderophores in iron-limiting conditions. The remaining isolates were found to be medium or low siderophore producers. These results agree with the established view that siderophores are produced in response to iron-starvation and the presence of freely available Fe^{2+} acts as a corepressor for the regulation of biosynthesis of siderophores (Bagg and Neilands, 1987).

Under iron-rich conditions, with 2μM Fe^{2+} added, most of the clinical isolates continued to synthesize considerable amounts of siderophores ($A_{630} < 1.00$) but this effect was not noticed in the environmental isolates suggesting that clinical isolates have a higher iron requirement than the environmental isolates. Clinical isolates were consistently found to produce higher mean levels of siderophores than the environmental isolates under both iron-deficient and iron-rich conditions at 28°C and also 37°C. These results demonstrate a clear difference in the siderophore producing ability of the isolates from the two different sources. The basis for the increased production of siderophores in clinical isolates compared to environmental isolates needs further study since they differ in their siderophore producing ability. The amount of iron available for bacteria in body fluids is very limited (Weinberg, 1978; Weinberg

1984; Weinberg, 1992a; Weinberg, 1992b; Weinberg, 1993). The ability of the clinical isolates to produce higher levels of siderophores will certainly help them to acquire iron and to ensure their survival in the host. However, three environmental isolates also did produce high levels of siderophores compared to a large number for the clinical isolates. These environmental isolates with high siderophore producing characteristics may be pathogenic.

The siderophore production of clinical isolates was consistently lower at 37°C than 28°C under both iron-limited and iron-rich conditions. However, Mateos *et al.* (1993) has reported that more hemolysins and cytotoxins were produced at 37°C, compared to 28°C, but protease activity decreased at this temperature. Nonetheless the differential production of siderophores at these two temperatures were found to be statistically non-significant suggesting that the temperature has little effect on its production.

Some interesting differences were evident in the plasmid profile of the clinical and environmental isolates of *A. hydrophila*. In general, the environmental isolates harboured more plasmids than clinical isolates. Very few plasmids in 5-20 Mda range were found in environmental isolates compared to the clinical isolates, suggesting a difference in their ability to acquire plasmids. Acridine orange efficiently cured all plasmids greater than 5.0 Mda by treatment for 24h but smaller (<5.0 Mda) plasmids were more resistant to treatment. Although environmental strains harboured more plasmids below 5.0 Mda the difficulty experienced in curing these plasmids from environmental isolates could not be explained on this basis alone since 8 plasmids of

the same size range were cured in clinical isolates. A prolonged treatment to remove the plasmids below 5.0 Mda was not attempted because of the possible damaging effect of acridine orange on chromosomal DNA.

No definite pattern of increase or decrease in siderophore production was obtained between the normal isolates and their cured derivatives. In general though, there was a small overall non-significant increase in the siderophore activity of the cured isolates compared to the normal isolates at the various conditions tested. Moreover, there was no correlation between the presence or absence of the plasmids with the production of siderophores. However, Borrego *et al.* (1991) found that 60% of the *A. hydrophila* isolates tested lost their siderophore activity after curing. The differences might be due to the techniques used for the detection of siderophores. We employed the quantitative CAS assay technique in which small differences in the siderophore production was readily detectable but Borrego *et al.* (1991) used the CAS agar plate method which only provides a qualitative result. Since no plasmid encoded gene(s) for siderophore production in *A. hydrophila* has/have been identified so far, the results of this study agree with the current view that genes for catecholate siderophore systems are chromosomal in this species (Barghouthi *et al.*, 1989; Barghouthi *et al.*, 1991).

Siderophore production of the cured clinical and environmental isolates remained unaffected by the curing experiments. Temperature had little effect on siderophore production although a minor mean decrease was observed at 37°C for both normal and cured clinical isolates. As noted in normal isolates the siderophore

production of the cured isolates were significantly increased under iron-limited conditions and also the cured isolates showed a significant increase in the siderophore activity of the clinical isolates compared to the environmental isolates. That is, the siderophore producing pattern remained unaltered in the plasmid cured derivatives. These observations further support the view that plasmids of *A. hydrophila* have little effect in the siderophore biosynthesis (Naidu and Yadav, 1997).

5.2 Siderophore production in *Vibrio cholerae*

All the *V. cholerae* isolates used in this study demonstrated siderophore activity when cultured under iron-limiting conditions at both 28°C and 37°C. As expected the siderophore production of the isolates increased dramatically under iron-limiting conditions compared to iron-rich conditions. These results are in agreement with the current view that the *V. cholerae fur* gene requires iron as a cofactor for the negative regulation of siderophore biosynthesis (Goldberg *et al.*, 1990a; Goldberg *et al.*, 1990b; Litwin *et al.*, 1992).

The production of siderophores varied between cultural conditions at 28°C and 37°C, and an increased siderophore activity was observed at 37°C compared to 28°C. The Student's one-tailed t-test analysis showed that there is a significant difference in the siderophore activity of the isolates at 28°C compared to the high value at 37°C. Since these isolates were obtained from the stool samples of local cholera patients, the observation on temperature may reflect that these *V. cholerae* isolates were adapted for survival and resistance in humans. Since *V. cholerae* is not an invasive pathogen

and can obtain nutrients rich in iron from the gut itself (Finkelstein, 1973; Wachsmuth *et al.*, 1994), it is doubtful whether siderophore production *in vivo* aids in the survival or pathogenicity of *V. cholerae*. Sigel *et al* (1985) has reported that vibriobactin, the siderophore produced by *V. cholerae*, is not a virulence factor in this organism. The fact that the *V. cholerae* isolates also did produce high levels of siderophores at 28°C could imply that siderophores could play an important role in salt and brackish water environments where the availability of iron is much reduced (Sigel and Payne, 1982).

5.3 Comparison between the sideropore production in *A. hydrophila* and *V. cholerae*

Both the *A. hydrophila* and *V. cholerae* strains are known to produce catecholate siderophores which are chromosomally encoded (Barghouthi *et al.*, 1989; Barghouthi *et al.*, 1991; Sigel and Finkelstein, 1978; Griffiths *et al.*, 1984; Litwin *et al.*, 1992). Since all the *A. hydrophila* and *V. cholerae* isolates tested exhibited siderophore activity, siderophore production can be considered a common feature in these two species, and is one of the main iron acquisition mechanisms. Although Borrego *et al.* (1991) reported that plasmids could play a role in siderophore production in *A. hydrophila*, we found no evidence for such a role for the plasmids. Siderophore production did not decrease in isolates which were cured of plasmids.

As expected the siderophore production of both *A. hydrophila* and *V. cholerae* isolates increased significantly under iron-limiting conditions regardless of the growth temperature. The clinical *A. hydrophila* isolates consistently produced higher amounts

of siderophores at 28°C than 37°C although this increase was found to be statistically non-significant. Under iron-limiting conditions the *V. cholerae* isolates were found to produce significantly higher levels of siderophore when grown at 37°C than 28°C. Further research is needed to determine the effect of temperature on the siderophore producing ability of the *A. hydrophila* and *V. cholerae* isolates.

5.4 Hemolysin production in *A. hydrophila*

Since almost half of the *A. hydrophila* isolates were non-hemolytic, hemolytic activity could not be considered a common feature of *A. hydrophila* isolates. It has been shown that all *Aeromonas* strains, regardless of their species designation and source of isolation, possess a β-hemolysin gene (Singh and Sanyal, 1992). They proved that non-hemolytic and α-hemolytic *Aeromonas* spp switched to production of β-hemolysin when passaged through rabbit ileal loops and subsequently reverted to their original hemolytic pattern when repeatedly subcultured or stored in the laboratory. This leads to the possibility that the beta-hemolytic gene operates in a repression and derepression mode and that the rabbit ileal loop passage removes this repression. This was evident from the study carried out by Husslein *et al.*, (1988), where it was found that sequences that are homologous to the β-hemolysin gene are present in all species of *Aeromonas*.

Hemolysin production of the clinical and environmental isolates of *A. hydrophila* increased significantly under iron-deficient condition compared to iron-rich

conditions. These observations point to a possible role for the hemolysin of *A. hydrophila* in iron acquisition. Hemolysin was also known to possess cytotoxic activity with ability to lyse various cell lines besides erythrocytes (Asao *et al.*, 1984). Gray *et al.*, (1990) found that there was a significant correlation between the production of a heat-labile cytotoxin and β-hemolysin. This was confirmed by the observations of Cumberbatch *et al.*, (1979) who postulated that these activities were either the repression of the same protein or subjected to the same genetic control. The erythrocytes and other vertebrate cells are rich in their iron content (Deis, 1983; Theil, 1987). The ability of hemolysin to lyse them could release their internal iron stores and circulate them in blood and body fluids of vertebrates (Valvano *et al.*, 1986; Gruenig *et al.*, 1987; Goebel *et al.*, 1988). But most of the iron released in this manner is complexed with host iron-binding proteins such as heme, hemoglobin, transferrin and lactoferrin (Bezkorovainy, 1987; Weinberg, 1984; Crichton and Charloteaux-Wauters, 1987; Morgan, 1981). Subsequently, the iron bound to these proteins are internalized by the bacteria through the mediation of heme-binding or lactoferrin- and transferrin-binding proteins displayed on the bacterial cell surface (Henderson and Payne, 1993; Chen *et al.*, 1996; Maciver *et al.*, 1996; Gray-Owen and Schryvers, 1995; Schryvers and Lee, 1989; Carroll *et al.*, 1996; Aebi *et al.*, 1996). Since *A. hydrophila* were known to be invasive pathogens of humans and also other vertebrates (Trust and Chapman, 1979; Harris *et al.*, 1985) where the availability of iron would be very limited (Weinberg, 1992a; Weinberg, 1993), the increased production of hemolysin in iron-deficient conditions could promote the survival of this species in iron-restricted tissues, and thus aid in the process of infection. This view is supported by Goebel *et al.*

(1988) who has reported that aerolysin synthesis by *Aeromonas sobria* increased under iron-limited conditions.

Riddle *et al.* (1981a) have reported that the presence of iron in growth media had an inhibitory effect on the synthesis of hemolysins. We found that, although an increase in the hemolysin production was demonstrated under iron-limited conditions, the presence of additional iron (100 μM) in the growth media did not alter the hemolysin production pattern of the isolates tested. This difference could be attributed to the different culture media used. We used Trypticase Soy Broth (TSB) with the addition of EDDA as the growth media but Riddle *et al.* (1981a) used minimal salts medium (MM). Nonetheless, these two studies point towards a significant role for iron in regulating the hemolysin production in *A. hydrophila*.

The hemolysin production in *A. hydrophila* appears to be linked to the growth temperature. The clinical isolates were found to produce significantly higher levels of hemolysin titers at 37°C compared to 28°C under both iron-limiting and iron-rich conditions. Similar observations were reported by Mateos *et al.* (1993) who found that human isolates exhibited higher hemolytic activity when cultured at 37°C than 28°C. This may indicate adaptation of the hemolysin production mechanisms in the pathogenicity of *A. hydrophila* for homeotherms. The growth temperature did not exert an effect on the hemolysin synthesis by the environmental isolates. Mateos *et al.* (1993) also found that the clinical isolates produced more cytotoxins at 37°C than 28°C. Since hemolysins were also known to possess cytotoxic activity (Asao *et al.*, 1984; Burke *et al.*, 1981; Rose *et al.*, 1989), the findings of our study strengthens the

view that growth temperature plays an important role in regulating the hemolysin production of the clinical isolates of *A. hydrophila.*

Aerolysin (β-hemolysin) has been shown to be a contributing factor in the pathogenesis of *A. hydrophila* infections (Allan and Stevenson, 1981; Rahim *et al.*, 1984; Stelma *et al.*, 1986). Another report by Gautam *et al.* (1992) indicates that although hemolysins can be one of the factors involved in the pathogenicity of enterotoxigenic *Aeromonas* strains, they are not required for the virulence of all *Aeromonas* spp. A similar conclusion was also reached by Santos *et al.* (1985)

A significant association between the hemolytic and cytotoxic activities of *A. hydrophila* was reported by Burke *et al.* (1981). This was confirmed by a recent report by Vadivelu *et al.* (1995) who found that hemolysin and cytotoxin production was closely related in all clinical isolates of *A. hydrophila.* Asao *et al.* (1984) demonstrated that the hemolysin also had cytotoxic activity and thus could be associated with the same protein. Further evidence was provided by Rose *et al.* (1989) who has reported that the hemolytic, cytotoxic and enterotoxic activities could be the toxic effects produced by a single polypeptide. Similar conclusions were drawn by Yadav *et al.* (1992) who found 100% association between the hemolytic and cytotoxic activity of the clinical *A. hydrophila* isolates tested.

The environmental isolates showed higher hemolytic titers than clinical isolates under all the conditions tested. Similarly Burke *et al.*, (1984b) reported that the hemolytic activity differed in clinical isolates compared to the environmental isolates.

Interestingly, Asao *et al.* (1986) found that the hemolysin produced by environmental isolates was immunologically and physicochemically different from that of clinical isolates. This difference could be attributed to the diversity in the amino-acid sequence of the hemolysin (Asao *et al.*, 1986). It has also been reported that virulence did not strictly correlate with the quantity of β-hemolysin elaborated, whereas some non-hemolytic strains when injected intraperitoneally were able to kill mice (Janda *et al.*, 1985; Brenden and Janda, 1987).The role of hemolysins of *A. hydrophila* outside the host animal is still uncertain and the basis for the increased production of hemolysin by the environmental isolates needs further study.

5.5 Hemolysin production in *V. cholerae*

Most of the *V. cholerae* isolates used in this study turned out to be non-hemolytic. These isolates have been confirmed as *V. cholerae* O1 biotype El Tor by hemagglutination and Voges-Proskauer tests (Cheeseborough, 1984). Previously the El Tor strains were found to be hemolytic while classical strains were not (Honda and Finkelstein, 1979). The detection of hemolysin production is not recommended for differentiating *V. cholerae* biotypes since hemolysin production by El Tor strains is not considered a consistent feature. Most of the *V. cholerae* O1 El Tor strains isolated nowadays are found to be hemolysin deficient (Roy and Mukerjee, 1962; de Moor, 1963; Dr. John J. Mekalanos, personal communication, Jan 13, 1997). The observations made from our study on the hemolysin production of local *V. cholerae* El Tor strains confirm this phenomenon.

It has been shown that the classical strains of *V. cholerae* possess a gene homologous to the El Tor hemolysin (Brown and Manning, 1985). But only the aminoterminal domain of this gene is expressed in the classical strains and this protein is non-hemolytic but possesses cytotoxic activity (Alm *et al.*, 1991). Interestingly, Singh and Sanyal (1992) have reported that both hemolytic and non-hemolytic *Aeromonas* strains possess a β-hemolysin gene. The *A. hydrophila* hemolysin was also known to possess cytotoxic activity (Asao *et al.*, 1984; Burke *et al.*, 1981; Rose *et al.*, 1989). These similarities between the hemolysins of *A. hydrophila* and *V. cholerae* may indicate the existence of similar regulation mechanisms for the expression of these toxins.

The hemolysin production was affected by the growth temperature. The hemolysin production was totally inhibited when the isolates were cultured at 37°C. At 28°C, some strains exhibited low hemolytic activity, while the majority was non-hemolytic. The hemolytic activity did not increase under iron-limiting conditions. In fact, less hemolysin production was detected under iron-limiting condition compared to iron-rich conditions. These observations rule out the possible role(s) of hemolysins of *V. cholerae* in iron acquisition. There are some differences between these results and those reported by Stoebner and Payne (1988). These authors have found that hemolytic activity increased in iron-deficient cultures of *V. cholerae* El Tor strains. This may indicate that there are variations in the hemolysin producing pattern of the El Tor strains and the regulation of the production of these toxins.

5.6 Comparison between the hemolysin production of *A. hydrophila* and *V. cholerae*

The *A. hydrophila* isolates produced hemolysins at both 28°C and 37°C while among the *V. cholerae* isolates low hemolytic activity was detected only at 28°C. Most of the *A. hydrophila* were medium and high producers of hemolysin at both temperatures whereas only low hemolysin producers were detected among the *V. cholerae* isolates at 28°C. This indicates that the *V. cholerae* strains are weak producers of hemolysin compared to the *A. hydrophila* isolates. The clinical isolates of *A. hydrophila* showed higher hemolytic activity at 37°C compared to 28°C while the clinical *V. cholerae* isolated exhibited hemolytic activity only at 28°C. These results could indicate that although growth temperature has a role in regulating the hemolysin production in both *A. hydrophila* and *V. cholerae*, there are some differences in how this is achieved in these two species.

Increased levels of hemolytic activity was detected among the *A. hydrophila* isolates under iron-limiting conditions, whereas the hemolytic activity of the *V. cholerae* isolates actually decreased under iron-limiting conditions. These observations indicate that while the hemolysins of *A. hydrophila* has a significant role in the iron-acquisition process, this was not the case with *V. cholerae*. Although it has been reported previously that hemolysins of *V. cholerae* play a role in the iron-acquisition process (Stoebner and Payne, 1988), this is doubtful since the El Tor strains isolated nowadays are mostly non-hemolytic (Roy and Mukerjee, 1962; de Moor, 1963; Dr. John J. Mekalanos, personal communication, Jan 13, 1997).

5.7 Protease production in *A. hydrophila*

The proteases of *A. hydrophila* have been suggested as virulence factors (Thune *et al.*, 1982). All the clinical isolates and 87.5% of the environmental isolates were proteolytic, suggesting that protease is an important survival characteristic of the *A. hydrophila* isolates. Similarly, Mateos *et al.*, (1993) found that 95% of the *A. hydrophila* strains studied showed proteolytic activity. In another study (Nieto and Ellis, 1991), it was found that all the ten isolates studied were positive for protease production. Several researchers found that although most *A. hydrophila* strains produce extra-cellular proteases, the amount of proteases produced varies considerably between strains (Riddle *et al.*, 1981b; Shotts *et al.*, 1985; Nord *et al.*, 1975).

Protease production in *A. hydrophila* was not affected by the availability of iron either at 28°C or 37°C. Although it has been reported previously that the presence of iron in culture media inhibited or decreased protease synthesis in this species (Riddle *et al.*, 1981a), in our study it was observed that the presence of iron did not decrease the protease production. The protease activity also did not increase in the absence of iron in culture media or in the presence of iron chelators such as EDDA. This could indicate that proteases of *A. hydrophila* do not play a role in the iron acquisition process.

The clinical isolates produced higher levels of proteases at 28°C compared to 37°C. The environmental isolates also showed a non-significant increase in their protease production at 28°C than 37°C. These results are identical to those reported by Mateos *et al.* (1993). These authors found that protease activity of the clinical strains decreased at 37°C compared to 28°C. These results indicate that growth temperature may influence the protease production of the *A. hydrophila* strains. The protease production of the clinical isolates also could be under tighter control by the growth temperature than the environmental isolates.

The clinical isolates consistently exhibited higher protease activity under all the various conditions examined. These observations point towards the possibility that the clinical and environmental strains differ in their protease producing ability.

5.8 Protease production in *V. cholerae*

The *V. cholerae* O1 hemagglutinin/protease was found to be immunologically and functionally related to *Pseudomonas aeruginosa* elastase (Hase and Finkelstein, 1990). It has also been reported that zinc and iron regulate the elastase production in *P. aeruginosa* at the translational level, although they probably use separate mechanisms (Brumlik and Storey, 1992).

All but two of the *V. cholerae* isolates used in this study exhibited protease activity at both 28°C and 37°C. One isolate which was proteolytic at 28°C failed to produce any protease at 37°C. Protease production did not increase under iron-limited

conditions. In fact, a significant increase in the protease production was noted under iron-rich conditions. This leads to the possibility that iron could positively regulate the synthesis of proteases in *V. cholerae*. At the same time, these results also indicate that proteases of *V. cholerae* have little role in the acquisition of iron. Since there is no significant difference between the protease production at both 28°C and 37°C, it can be concluded that growth temperature has no effect on the protease production of *V. cholerae* strains.

5.9 Comparison between the protease production in *A. hydrophila* and *V. cholerae*

Since most of the *A. hydrophila* and *V. cholerae* isolated used in this study exhibited protease activity, it can be safely concluded that protease production is a normal feature in these two organisms. The proteases of *A. hydrophila* were not affected by the absence or presence of iron, while a general increase was observed in the protease production of the *V. cholerae* isolates as the iron concentration increased. This suggests that the proteases produced by these two species do not have a role in the iron acquisition process.

The protease production in *A. hydrophila* was affected by growth temperature with higher protease production at 28°C while the protease production in *V. cholerae* remained generally unaffected by the growth temperature. The basis for this differential regulation by growth temperature is currently unknown and needs further study.

5.10 Siderophore, hemolysin and protease production in *A. hydrophila*.

All the clinical and environmental isolates produced siderophores under all the conditions tested. Similarly, all the clinical isolates turned out to be protease producers, while only two of the 14 environmental isolates tested negative. From these observations it can be ascertained that siderophore and protease production are universal phenomena in *A. hydrophila*. In contrast, almost half of the clinical and environmental isolates of *A. hydrophila* produced negative results for hemolytic activity. Hence, hemolysin production can be considered not a reliable or consistent feature in *A. hydrophila*.

When cultured under iron-limiting conditions the siderophore and hemolytic activity of both the clinical and environmental isolates increased significantly, while this effect was not noticed for protease production. Siderophore production has been established as one of the main iron acquisition mechanisms of many microbes including *A. hydrophila* (Woolridge and Williams, 1993; Barghouthi *et al.*, 1989; Massad *et al.*, 1991; Zwyno *et al.*, 1992). Although several bacteria were known to utilize heme compounds as their iron source, this has not been established conclusively for *A. hydrophila*. Since hemolysins are capable of lysing erythrocytes and other cells which are rich in iron content (Howard and Buckley, 1985; Ikigai *et al.*, 1996; Song *et al.*, 1996; Menzl *et al.*, 1996), a role for hemolysins in iron acquisition process has been suggested. The increased levels of hemolysin production under iron-limiting conditions obtained from this study, suggests such a role for the hemolysins of *A. hydrophila* and also that this species are capable of utilizing heme compounds as their iron source.

Since *A. hydrophila* were known to cause bacteremia and septicemia (Trust and Chapman, 1979; Harris *et al.*, 1985), this possibility is not out of consideration. All the isolates possessed siderophore activity but half of the isolates were non-hemolytic. In view of this, the importance of hemolysins in the iron acquisition process should be secondary to siderophore production. These results clearly indicate that siderophore and hemolysin production are the two main mechanisms of iron acquisition in *A. hydrophila* and rules out such a role for the proteases of *A. hydrophila*.

The growth temperature was found not to have any significant effect on the siderophore production of both the clinical and environmental isolates. Similarly, the hemolysin and protease production of the environmental isolates of *A. hydrophila* were also not altered significantly at either 28°C or 37°C. But for the clinical isolates a significant difference was noted for both hemolysin and protease production. A higher hemolytic activity was observed at 37°C while protease activity decreased at this temperature. These findings are identical to those reported by Mateos *et al.* (1993). While the significant role of the growth temperature in influencing the hemolysin and protease production cannot be denied, the basis for this effect is unknown and need further investigation. Probably, the growth temperature affects the rate of enzyme synthesis or transport.

Clinical isolates (including the acridine orange treated / plasmid cured clinical isolates) produced more siderophores than the environmental isolates. Clinical isolates also produced higher levels of proteases than environmental isolates at 28°C while a non-significant increase was noted at 37°C. In contrast to the above results,

environmental isolates possessed more hemolytic activity than clinical isolates at both 28°C and 37°C. These results show that the growth temperature and the source of isolates significantly influence the synthesis of extra-cellular products in *A. hydrophila*. The basis for this differential production of siderophores, hemolysins and proteases in clinical and environmental isolates needs further study.

5.11 Siderophore, hemolysin and protease production in *V. cholerae*.

All the *V. cholerae* isolates produced siderophores the conditions tested at both 28°C and 37°C. All but two of the *V. cholerae* isolates exhibited protease activity at both 28°C and 37°C. One isolate was proteolytic at 28°C but not at 37°C. Thus it can be concluded that as was the case for *A. hydrophila*, siderophore and protease production are universal phenomena in *V. cholerae*. Surprisingly, all the *V. cholerae* isolates were non-hemolytic at 37°C while only 13 of the 38 isolates were low hemolytic at 28°C. All the *V. cholerae* isolates used in this study were of the El Tor biotype. El Tor strains were originally classified as hemolytic while classical strains were not (Honda and Finkelstein, 1979). Lately, this was not considered a reliable characteristic as non-hemolytic El Tor strains have been isolated (Roy and Mukerjee, 1962; de Moor, 1963; Cheeseborough, 1984; Dr. John. J. Mekalanos, personal communication, Jan 13, 1997). The results from our study confirms this view as most of the El Tor strains used turned out to be non-hemolytic.

As expected siderophore production of the *V. cholerae* isolates increased significantly when cultured under iron-limiting conditions. But no increases were noticed in the hemolysin and protease production under iron-limiting conditions. It could be suggested that siderophore production is the main, if not the only, mechanism of iron acquisition for *V. cholerae* strains used in this study.

So far, *V. cholerae* strains were not shown to acquire iron from host proteins, transferrin or lactoferrin. The ability of *V. cholerae* El Tor strains to obtain iron from heme and hemoglobin has been demonstrated (Stoebner and Payne, 1988; Henderson and Payne, 1993). It has also been reported that increased amounts of hemolysins were produced under iron-deficient conditions and thus a role for hemolysins in iron acquisition has also been suggested in these species (Stoebner and Payne, 1988). Since most of the El Tor strains isolated nowadays are non-hemolytic (Roy and Mukerjee, 1962; de Moor, 1963; Dr. John J. Mekalanos, personal communication, Jan 13, 1997), the importance of hemolysins in iron acquisition should be re-evaluated. Although in *Vibrio vulnificus*, an indirect role for protease in iron acquisition has been proposed (Nishina *et al.*, 1992), the results obtained from our study suggests that the proteases of *V. cholerae* are not involved in the iron acquisition process.

Growth temperature was found to influence the siderophore and hemolysin production of the isolates. Significantly higher amounts of siderophores were synthesized at 37°C compared to 28°C. No hemolysin production was noticed at 37°C, but some of the isolates exhibited low hemolytic activity at 28°C while the majority remained non-hemolytic. At the same time, growth temperature did not affect the protease production of the *V. cholerae* isolates. The basis for the effect of growth temperature on these extra-cellular products is unknown and need further study.

5.12 Comparison between the production of virulence-associated factors in *A. hydrophila* and *V. cholerae*.

Both the siderophore and protease production can be considered as consistent features in *A. hydrophila* and *V. cholerae* whereas hemolysin production is not. The main mechanism of iron acquisition for *A. hydrophila* and *V. cholerae* is siderophore mediated iron acquisition. As the hemolysins of *A. hydrophila* were also found to increase under iron limited conditions it could be suggested that hemolysins play a role in the iron acquisition process of the hemolytic strains of *A. hydrophila*. But this was not the case for *V. cholerae* as most of the isolates were non-hemolytic and no increases in the hemolysin production were noted in the positive isolates. The proteases of both *A. hydrophila* and *V. cholerae* did not increase under iron-limited conditions indicating that they have no role in iron acquisition in these bacteria.

While both siderophores and hemolysins are involved in the iron acquisition process in *A. hydrophila*, siderophore production is the only mechanism of iron acquisition for the *V. cholerae* El Tor isolates used in this study. Whether this is true for all the El Tor strains isolated worldwide is currently not known.

Siderophore production in *A. hydrophila* was not significantly affected by growth temperature although a slight increase was noted at 28°C compared to 37°C. But in *V. cholerae* a significant increase in the siderophore production was obtained at 37°C than 28°C. The clinical isolates of *A. hydrophila* showed increased hemolysin production at 37°C while the environmental strains remained unaffected by the growth

temperature. Growth temperature was also seen to affect the hemolytic activity of the *V. cholerae* isolates, where 13 of the 38 isolates were hemolytic at 28°C while none of them were hemolytic at 37°C.

The clinical isolates of *A. hydrophila* showed significantly higher protease production at 28°C than 37°C while for the environmental isolates this difference was not significant. In contrast, the *V. cholerae* isolates showed no significant difference in the protease production either at 28°C or 37°C.

From these observations it can be ascertained that the production of virulence factors in *A. hydrophila* and *V. cholerae* were influenced to various extents by the growth temperature. In the case of *A. hydrophila*, the effect of growth temperature also seems to be dependent upon the source of the isolates, since clinical isolates produced significantly higher amounts of hemolysins and proteases at 37°C compared to 28°C, but this effect was not significant ($p > 0.05$) for the environmental isolates.