A GENETIC AND PHYSIOLOGIC APPROACH TO THE CHARACTERIZATION OF THE PJM1 VIRULENCE PLASMID, A MEDIATOR OF IRON SEQUESTRATION IN *VIBRIO ANGUILLARUM* 775

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MARY ANN WALTER

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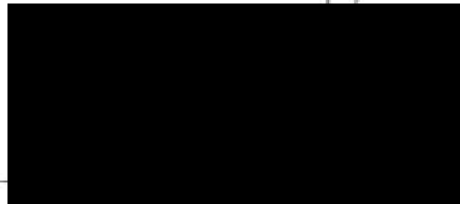

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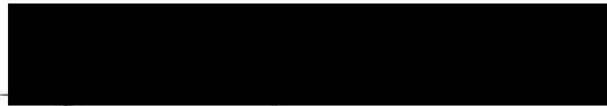

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I've Been On A Journey

My journey has been long and cold
My being lost and found
A warm release I beg to find
Within my soul, within my mind.

I've given of flesh and received of flesh
I've watched my toil and given less.
Belief in love is rarely seen
I will find this in my dreams.

Dreams to build a mountain high
Of beauty, truth, and lullabies
For harmony, joy, and peace within
As I prepare for my journey again.

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I wish to sincerely thank my major professor Dr. J.H. Crosa for understanding "my type" of love affair with science, for his never failing faith in my abilities, and his dedication to science,

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may I return the light of wisdom, truth, and love to all.

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ABBREVIATIONS

Genes

ade	-adenine biosynthesis
cir	-colicin I resistance
ent	-enterobactin biosynthesis
exb	-iron deficiency (over production of enterochelin)
fec	-ferric citrate uptake
fep	-ferric enterobactin uptake
fes	-ferric enterobactin esterase
fhu	-ferric hydroxamate uptake
fur	-ferric uptake regulation
gua	-guanine biosynthesis
iuc	-iron uptake chelator
iut	-iron uptake transport
lac	-lactose biosynthesis
ton	-T one phage receptor

Restriction endonucleases

HI	-*Bam*HI
ClaI	-*Cla*I
RI	-*Eco*RI
HIII	-*Hind*III
PvuII	-*Pvu*II
XI	-*Xho*I

Symbols

Ap	-ampicillin
BHI	-brain heart infusion medium
DHBA	-2,3-dihydroxybenzoic acid
DNA	-deoxyribonucleic acid
EDDA	-ethylenediamine-di (o-hydroxyphenyl acetic acid)
EDTA	-(ethylenedinitrilo)-tetraacetic acid
EPR	-electron paramagnetic resonance
Fe	-iron
Km	-kanamycin
Ksol	-solubility constant
LD_{50}	-mean lethal dose
NAD(P)H	-nicotinamide-adenine-dinucleotide-phosphate
Nal	-nalidixic acid
Nm	-neomycin
PPi	-inorganic pyrophosphate
r	-resistance
RNA	-ribonucleic acid
s	-sensitive
SDS-PAGE	-sodium dodecyl sulfate polyacrylamide gel electrophoresis
Str	-streptomycin
Tc	-tetracycline
Tn	-transposon
Rif	-rifampicin

Units of Measurements

°C	-degrees Centigrade
d	-daltons
g	-gram
h, hr	-hour
K	-thousand
kb	-kilobase pair
M	-molar
mA	-milliamperes
Md	-megadalton
min	-minute
pmoles	-picomoles
s	-seconds
V	-volts

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

A variety of bacteria produce infectious diseases of fish resulting in major economic loss among commercially important species. Controlling infectious diseases of fish remains the single most important factor in the success of aquaculture. *Vibrio anguillarum* 775, a marine fish pathogen which causes vibriosis (a terminal hemorrhagic septicemia in fish), carries a 65 kilobasepair (kb) plasmid (pJM1) that is necessary for virulence. This plasmid also mediates iron sequestration by the bacteria. Despite an extensive literature on the disease process vibriosis, the molecular basis for this important disease is not understood. Therefore, my thesis project has been involved with the characterization of the pJM1 virulence plasmid mediator of iron sequestration in *Vibrio anguillarum* 775 from a genetic and physiologic approach.

Derivative plasmids of pJM1 were generated using transposition mutagenesis with the TnA transposon Tn*1* donated from the plasmid RP4. Host bacteria containing certain of the derivative plasmids were changed in their ability to sequester iron and cause disease. That is, those bacteria unable to obtain iron due to a Tn*1* insertion into the pJM1 plasmid or a subsequent deletion of the pJM1 plasmid due to a Tn*1* insertion/deletion event were avirulent as determined in a salmonid fish model system. Mutants unable to grow under conditions of iron limitation were further screened in a physiologic bioassay system able to distinguish those strains deficient in a siderophore activity and/or a receptor activity for a ferrisiderophore complex. Two different types of mutants were found, one class having a siderophore activity minus receptor

activity positive phenotype and the other class a siderophore and receptor activity minus phenotype. Physiologic comparison studies have shown that other species of Vibrio were not able to reverse the growth inhibition of iron stressed V. anguillarum 775; however, some strains of V. anguillarum (other than 775) have been found to cross-feed V. anguillarum 775 iuc mutants as well as iuc, iut mutants and the isogenic plasmidless V. anguillarum.

Restriction endonuclease analysis of the plasmid using the various Tn1 insertions has allowed me to map the iron sequestration region of the pJM1 plasmid to an area containing BamHI fragment 1 and possibly parts of BamHI fragments 5 and 6 which border BamHI fragment 1. Eight BamHI fragments ranging in size from 19.9 kb to 2.1 kb were generated from a complete digestion of the pJM1 plasmid and 4 SalI fragments ranging from 29.7 kb to 1.4 kb.

There are similarities in the pathogenesis of vibriosis in marine fish and the invasive septicemic diseases in man and domestic animals. The system that most closely parallels the iron sequestration capabilities of the pJM1 virulence plasmid is the pColV-K30 plasmid in Escherichia coli. To investigate the similarity at the genetic level of the pColV-K30 and the pJM1 plasmids with respect to iron sequestration, cloned regions involved in iron uptake were used to determine DNA homology between the two systems. We found an extensive lack of homology between the pColV-K30 and the pJM1 plasmids with respect to their iron sequestration regions; moreover, using a physiologic bioassay no cross-feeding between the two systems was found.

The objectives of this thesis project were:

1. Generate *Vibrio anguillarum* mutants in iron sequestration through the transposon Tn*1* insertion into the pJM1 plasmid.
2. Generate a restriction endonuclease map of the pJM1 plasmid.
3. Generate a restriction endonuclease map of the Tn*1* insertions with their orientation on the pJM1 plasmid.
4. Generate comparison studies on the iron sequestration properties of *Vibrio anguillarum* 775 and its pJM1 plasmid through physiologic and genetic comparison with a similar virulence plasmid pColV-K30 and a physiologic comparison of other *Vibrio* species and *V. anguillarum* strains.

II. LITERATURE REVIEW

A. The Pathogenic Vibrio

The type genus of the family *Vibrionaceae* is *Vibrio* (Pacini, 1854). The genus *Vibrio* (Fig. 1) contains short asporogenous curved or straight rods, 0.5 by 1.5-3.0 μm, and is motile by means of a single polar flagellum (with some exceptions). *Vibrio* are Gram-negative, non-encapsulated facultative anaerobes, that ferment glucose without forming gas and are mostly oxidase-positive (Shewan and Veron, 1974). The genus *Vibrio* is closely related to the genera *Aeromonas*, *Photobacterium*, *Lucibacterium*, *Plesiomonas*, and *Pseudomonas*. The genus *Vibrio* contains some of the most important intestinal pathogens of man including the cause of epidemic Asiatic cholera, *Vibrio cholerae* (Trevisan, 1885; Lankford, 1959). The cholerae bacillus, O-group 1, is separated by O-group 1 antiserum positive agglutinability from the "non-agglutinable vibrios" (NAGs) or the "non-cholera vibrios" (NCVs) which include the species *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. fluvialis*. These species have all been found to cause disease in man (Blake et al., 1980). *Vibrio cholerae* is the type species, a human pathogen that causes a diarrheal (profuse, watery diarrhea with no inflammatory cells) disease by interaction of the cholera bacillus at the site of the small intestine. Man, and his contaminated water supply, are the only major reservoirs of infection. The bacteria attach to mucosal cells, rarely penetrating, and multiply on the epithelial cell surface. They induce disease by producing a potent enterotoxin (Finkelstein, 1973) with extraintestinal manifestations of dehydration, shock, and hypokalemic nephropathy. *V. parahaemolyticus*,

another intestinal pathogen, is the leading cause of diarrheal disease in Japan (Miwatani & Takeda, 1976.) This marine organism, which inhabits estuaries and other coastal waters throughout the world, is a major cause of gastroenteritis involving seafood. It appears to be transmitted exclusively by food, usually raw or cooked seafood and has been reported to have a nine minute generation time under ideal conditions enabling the organism to multiply very rapidly in mishandled foods (Katoh, 1965). V. alginolyticus and V. vulnificus are two human pathogens associated with wound infections and septicemia (Baumann et al., 1973; Blake et al., 1980; Pezzlo et al., 1979). V. alginolyticus was not believed to be a human pathogen until 1973 when six tissue infection samples were identified to be V. alginolyticus by Zen-Yoji et al. (1973). This organism has also been recovered from ears (acute otitis media patients), blood, cutaneous ulcers, and burn patients. V. vulnificus was earlier referred to as the lactose fermenting (L+) Vibrio (Hollis et al., 1976). Usual victims have had contact with seawater or saltwater crabs; moreover, patients with septicemic onset appeared to have been infected by eating raw oysters (Blake et al., 1979). The median incubation periods were 12 hr for wound infections and 16 hr for primary septicemias. V. fluvialis, previously called Group F (EF6), have been reported to cause a cholera-like disease in humans (Blake et al., 1980; Huq et al., 1980; Lockwood et al., 1982; Tacket et al., 1982) except that some patients had blood and mucus in their stools and some abdominal pain and fever. V. anguillarum, the organism of primary interest in this author's research project, is a pathogen of salmonid fishes that causes a terminal hemorrhagic septicemia with mortalities approaching 100 percent if the disease is left unchecked (Fryer et al., 1972; Rucker et al.,

1953). It was first described as the cause of "red pest of eels" in the Baltic Sea by Bergman (1909) and later found to also attack cod, Gadus morhua, (Bergman, 1912). The quest for the mechanism used by this bacterium to cause disease has led to an investigation of its ability to sequester iron from its enviroment (Crosa, et al., 1977; Crosa, 1980; Crosa, et al., 1980; Walter, et al., 1983). The next section discusses iron and virulence.

B. Iron Acquisition And Its Role In Bacterial Virulence

Many iron containing compounds are found in living cells. The iron-containing electron transfer compounds, such as ferredoxins and cytochrome oxidases, as well as the iron-containing oxygenases and hydroperoxidases all play essential roles in the basic metabolism of many cell types. Moveover, iron is an essential component of ribotide reductase, an enzyme with a key role in the synthesis of deoxyribonucleic acid (DNA). Thus, in view of the many and varied requirements for iron in each cell, it might be expected that there would be multiple systems for the acquisition of iron. This section of the literature review will focus on how bacteria sequester the essential nutrient iron from their environment and how the ability to compete successfully for iron from a host enables a disease state.

Most if not all bacteria require iron for growth (Waring & Werkman, 1942) with the possible exception of the lactic acid bacteria (Neilands, 1972); however, iron in an aerobic environment is not readily available for assimilation by bacteria. Under physiologic pH, ferric ion (Fe^{3+}) forms an insoluble precipitate of iron hydroxide $Fe(OH)_3$, $K_{sol} = 10^{-38.7}M$ (Spiro & Saltman, 1969), which means that ferric hydroxide will tend to

polymerize and precipitate once the free (Fe^{3+}) concentration exceeds 10^{-18} M (Bullen et al., 1971). Moreover, bacteria attempting to colonize their hosts also face an iron shortage. The free iron concentration in blood is 10^{-18} M or less being in equilibrium with iron bound to the serum iron binding proteins transferrin (in serum) and lactoferrin (in secretions) (Bullen et al., 1978). These proteins bind iron with association constants as high as 10^{36} M and are only partially saturated. The presence of these unsaturated iron-binding proteins has been shown repeatedly to contribute to the bacteriostatic and bacteriocidal qualities of serum and secretions (Bullen et al., 1974; Sussman, 1974; Arnold et al., 1977). Thus, bacterial adaptation to the low amounts of available iron in body fluids is a prerequisite for pathogenicity (Bullen, 1981; Miles & Khimji, 1975). It is well known that there are several other factors that can affect virulence, such as invasion of tissues by bacteria, bacterial attachment, inhibition of phagocytosis, complement dependent bacterial killing, and toxin production (McCloskey, 1979). However, even though virulence is multifactorial, it can only be obtained once an organism has established growth. The question of how an organism can establish itself in an environment of free iron too low to allow growth has been investigated (Payne & Finkelstein, 1978; Weinberg, 1978). The adaptation of bacteria to low iron conditions can take at least three forms, 1. production of iron-chelating agents that can compete with partially saturated host iron-binding proteins for (Fe^{3+}) (Neilands, 1977), 2. uptake of heme compounds (Perry & Brubaker, 1979), and 3. activity with iron-binding factors on the bacterial cell wall itself (Simonson et al., 1982). However, in the presence of high levels of inorganic iron, cells

nonspecifically transport the iron into the cytoplasm. Little is known about how this system operates except that it is the one used by cells to assimilate iron when they are grown in complex laboratory media or in a media in which the iron concentration is several micromolar or higher.

There is evidence to suggest that iron overload or increased serum iron saturation predisposes human beings to infection (Caroline, 1974; Weinberg, 1974; Weinberg, 1975). It has been reported that people with kwashiorkor or acute myelogenous leukemia, two disease states that demonstrate high serum iron saturation, are more prone to bacterial infections (McFarlane et al., 1970; Caroline et al., 1969). Moreover, it has been known that during trauma injury the mammalian host is more susceptible to infection which may be due to lysed erythrocytes releasing heme compounds into the injured area. Thus, nonvirulent organisms, those usually unable to sequester iron from the host, are then able to grow and cause disease. This example has been well illustrated by the colonization of _E. coli_ in the peritoneal cavity after strangulation of the gut (Bullen, 1981). Moreover, it is believed that the heme that is coinjected with _Yersinia pestis_ (plague bacillus) after a flea bite allows this organism to initiate an infection in the otherwise iron-limiting environment of the host (Perry & Brubaker, 1979). Experimentation on the effect of added iron upon microbial infection has shown that iron added in sufficient quantity to double the saturation value of the host iron-binding proteins will have a marked effect on the ability of various microbial strains to multiply. A decrease in the inoculum size to achieve growth by four to five log units has been noted. Thus, it has been determined that in many cases, iron treated animals were more prone to infection from a variety of microorganisms

than were their untreated counterparts (Bullen et al., 1974; Sussman, 1974; Payne & Finkelstein, 1975; Sawatzki et al., 1983). Even though the actual role of injected iron has not been determined, the most popular notion is that injected iron simply and easily fulfills a nutritional requirement. However, procedures that reduce the concentration of iron (mice fed an iron-deficient diet) showed an increase in resistance to bacterial infection (Puschmann & Ganzoni, 1977).

When a bacterial cell is exposed to ferric iron limitation conditions (less than 1 μM) those cells that are able invoke a high affinity system of iron uptake. High affinity iron transport systems are composed of two parts 1. a soluble low molecular weight ferric-specific ligand called a siderophore (Lankford, 1973; Neilands, 1972; Neilands, 1973) and 2. a matching membrane receptor for the iron-siderophore complex (Neilands, 1981). The expression of this system is under the control of iron. Chemically, siderophores are generally all-oxygen, hexadentate ligands with a specificity for iron. Siderophores are virtually specific for Fe(III) with formation constants in the range of 10^{20} to 10^{50} M. Siderophores range in size from about 500 to 1000 d (daltons) which makes them too large to diffuse freely through the small water-filled pores of the Gram-negative outer membrane (Nielands, 1976). Most of the chemically identified siderophores fall into two classes, the catechols and the hydroxamic acids. The prototype siderophore of the catechol member of microbial iron carriers is enterobactin, a complex of cyclo-tri-2,3-dihydroxybenzoylserine that binds iron via three bidentate catechol groups yielding a hexacoordinate ferric complex (O'Brien & Gibson, 1970; Pollack et al., 1970; Neilands, 1973). The prototype hydroxamate is ferrichrome, a trihydroxamate complex of a

cyclohexapeptide (Emery & Neilands, 1961) binding to ferric ion via the bidentate hydroxamate group [-N(OH)-CO-] forming a hexacoordinate complex (Neilands, 1973). The second component of this high affinity system is the production of outer membrane protein receptors and any necessary enzymes involved in the uptake and release of iron from the ferric-ligands.

In response to bacterial infection the unified effect of all iron-restricting mechanisms in normal and immune animals has been called nutritional immunity (Kochan, 1973; Weinberg, 1974). Nutritional immunity can be defined as a host's ability to discourage microbial growth by producing a hypoferremia. Hypoferremia may occur by 1. decreasing the absorption of iron from the gastrointestinal tract, 2. increasing the production of iron-binding proteins, 3. suppressing the production or activity of siderophores and/or, 4. contraction of iron in the plasma compartment plus expansion of iron in the storage compartment (Kochan, 1975; Weinberg, 1978). As mentioned above, the injection of iron into infected animals overcomes this nutritional immunity and allows the unrestricted growth of the invading infectious microorganism (Rogers, 1973; Yancey et. al., 1979). The infection promoting effect of iron has shown that iron predisposes not only normal but also immune animals to bacterial infections (Kochan et al., 1978); moreover, iron was not found to interfere with the development of immunity, production of antibodies and their serological activities, or delayed hypersensitivity but was found to interfere with the expression of antibacterial responses in vaccinated animals (Kochan, 1983). Kochan et al. (1984) found the percent mortality in normal mice and immune mice (vaccinated with live avirulent S. typhimurium) were very different after 20 days, 40%

mortality for nonimmune mice but 0% mortality for immune mice. However, upon addition of iron with the test virulent organism there was no difference in mortality. Iron addition appears to not only serve as a nutrilite but also to neutralize the actively acquired immunity. Their findings also suggested that more iron was needed for neutralization of immunity than for satisfaction of the nutritional need of infecting bacteria. Using both live and killed vaccines of S. typhimurium, two antibacterial systems were found; one that was neutralizable by iron and can be induced by live vaccines from avirulent bacteria and the other that was resistant to iron and was induced in animals by a combined effect of live avirulent and killed virulent bacteria. Further studies on this system led the authors to conclude that iron-resistant immunity may be due to the synergistic action of phagocytes of immunologically stimulated animals and the action of a specific antibody that appears to be induced by an antigen common to virulent S. typhimurium. Thus, it appears that most students of this field agree that the efficiency with which the bacteria acquire iron in the body of the infected host is a significant factor in virulence (Kochan et al., 1977, 1984; Payne & Finkelstein, 1975; Weinberg, 1978).

C. Procaryotic Iron Sequestration Systems - An Overview

Many microorganisms excrete high affinity chelating agents, siderophores, in order to sequester iron from their environment (Neilands, 1981). Siderophore iron transport has been investigated, for one, by following the rate of uptake of radioactively labeled iron. Two basic mechanisms have been proposed for iron uptake from ferric siderophores using a ^{59}Fe- or ^{55}Fe-labeled iron and a ^{14}C- or ^{3}H-labeled

ligand. In the first, illustrated by ferrichrome transport by Ustilago sphaerogena (a smut fungus), both labeled iron and labeled ligand are taken up at an identical rate (Emery, 1971). In every case so far examined, removal of iron from the ferric ionophore (binds alkali-metal cations; that is, ferrichrome may be a ferric ionophore) inside the cell involves reduction of siderophore iron (III) to iron (II) and then the ligand reappears in the medium ready for further chelate formation (Cooper et al., 1978). Further studies with ferrichrome transport using isotope transport assays coupled with EPR (electron paramagnetic resonance spectroscopy) indicate that reduction of the iron in ferrichrome lags behind the rate of ferrichrome uptake suggesting that the chelate molecule is taken up without dissociation (Ecker et al., 1982). A version of this mechanism found the ferric siderophore entering the cell but the siderophore was destroyed upon iron release. An example of this was found with the enterobactin system (Emery, 1971; Cooper et al., 1978). In the second mechanism, the uptake of labeled iron occurred without the uptake of the ligand; that is, the siderophore brought the iron to the cell surface where dissociation occurred. Iron uptake via ferrichrome A (a second siderophore secreted by this smut fungus) in U. sphaerogena occurs by this "iron taxi" (Raymond & Carrano, 1979) mechanism; that is, iron from ferrichrome A was transported into the cell at a rate equal to the rate of disappearance of the EPR signal in the cell suspension (Ecker et al., 1982). Thus this "iron taxi" mechanism involves a reductive removal of iron at the cell surface rather than intracellular reduction as is the case for ferrichrome. Iron uptake from ferric exochelins in Mycobacterium smegmatis (Ratledge et al., 1982) and

ferric rhodotorulic acid in Rhodoturula pilimanae (Carrano & Raymond, 1978) also occur by the 'iron taxi' mechanism.

The requirement for a receptor for the ferrisiderophore complex follows from the observation that water-soluble compounds greater in size than 500 to 600 d cannot cross the Gram-negative bacteria's small water-filled pores (Nikaido, 1979). The function of a receptor is to bring the ferri-siderophore to, or through, the envelope of the organism where the iron is made available for the nutritional requirements of the cell. The actual first identification of a siderophore receptor was the ferrichrome receptor (Wayne & Neilands, 1975). This particular protein also acts as the attachment site for the phages T1, T5, and Ø80 and for colicin M and the antibiotic analogue of ferrichrome, albomycin (Neilands, 1982). It is believed that the 78,000 d outer membrane protein (tonA) visible on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) is the protein receptor or a protein component of the ferrichrome receptor complex. Both inner membrane and outer membrane proteins have been found involved with iron transport; however, the majority of iron-related proteins detected are in the outer membranes. Studies on carbohydrate transport in bacteria have found periplasmic proteins involved in binding or processing of transported solutes (Dills et al., 1980); however, no periplasmic proteins have been found to be involved in iron transport. Monosaccharides and amino acids, being small in molecular weight (below the exclusion limit of the Gram-negative outer cell envelope), will pass through the protective barrier with no apparent need for an outer membrane receptor. Thus one can see a different problem for the cell in the way it sequesters small nutrients and the way it acquires nutrients in a size too large for entry into the periplasmic

space. The molecular weights of the iron-related outer membrane proteins, about 80,000 d by SDS-PAGE analysis, are large enough to easily span the periplasmic space and contact the inner membrane to allow transport of iron into the cytoplasm. Bayer demonstrated by electron microscopy that the ferrichrome receptor is located at a Bayer adhesion zone (Bayer, 1968) perhaps allowing insight on the mechanism of iron transport from the extracellular space into the cytoplasm.

For those organisms that transport the entire ferrisiderophore it has been proposed that the release of iron may be accomplished by one of the three following mechanisms: (i) enzymatic reduction of iron, (ii) hydrolysis of the ferrisiderophore, and (iii) nonenzymatic transfer of iron to a second chelator (Arceneaux, 1983). It is generally thought that release of iron from ferrisiderophores by enzymatic reduction of iron to an acceptor or directly into a metabolic sequence is feasible since the ferrous iron is only weakly bound to siderophores (Neilands, 1973). Some studies, in support of this mechanism, have found ferrisiderophore reductase activities that catalyzed reduction of iron from various ferrisiderophores to ferrous acceptors in Pseudomonas aeruginosa (Cox, 1980A), Bacillus megaterium (Arceneaux & Byers, 1980), Micrococcus denitrificans (Tait, 1975), B. subtilis (Gaines et al., 1981; Lodge et al., 1980) and U. sphaerogena (Straka & Emery, 1979). Extensive studies have been performed on the mechanism of iron release by ferrichrome and enterobactin (Cooper et al., 1978). Despite the similarity in function and regulation of these two siderophores, the basic mechanism of iron release is different. With the hydroxamate chelates, after the intracellular iron was released, the iron-free ligand was secreted back into the medium to be reused (Emery, 1971).

Ferrisiderophore reductases may be an essential part of a bridge complex that makes receptor bound iron available to the cellular metabolism and thus one siderophore molecule can shuttle more than one iron ion into the organism. In contrast, the cyclic triester linkages of ferric enterobactin are cleaved by a specific esterase that results in the unusable cleavage product, 2, 3-dihydroxybenzoylserine (Emery, 1971). It was established that esterase activity is vital to enterobactin-mediated iron transport since esterase-deficient mutants are pink colored due to an accumulation of intracellular ferri-enterobactin and are growth limited under conditions of iron stress (Langman et al., 1972; Cooper et al., 1978). O'Brien et al. (1971) suggested that enterobactin cleavage was necessary because of the very low reduction potential of the ferric-enterobactin complex, lower than physiologically available; however, the ferric tris (2, 3-dihydroxybenzoylserine) complex is readily reduced electrochemically at potentials well within the physiological range (Cooper et al., 1978). This hydrolysis of ferric enterobactin must be necessary because the enterobactin binds the ferric iron so strongly [$Fe(III) + ent^{6-} = Fe(ent)^{3-}$, log K=52 (Hollifield & Neilands, 1978)] that the cytoplasmic milieu cannot simply reduce the iron off but must destroy the integrity of enterobactin after only one iron transport cycle. However, since enterobactin has such a high affinity for iron it is believed the bacteria may use this as a growth advantage by denying iron to microorganisms in the same environment. Simply stated, the microbial hydroxamate iron transport chelates are reduced at potentials near those of physiological reductants such as NAD(P)H but the extremely low redox potential of ferric enterobactin preclude this mechanism and thus the ligand is modified instead of the iron. The non-enzymatic route of iron

release has been predicted to require too much time under physiological conditions since the rate of incorporation of iron into macromolecules has been observed to be more rapid (Arceneaux & Byers, 1976) than the exchange between ferri- and deferri-compounds (Harris et al., 1979; Tufano et al., 1981).

Synthesis of many siderophores and some components of their transport systems studied to date is regulated. One gene has been found by mutant studies that coordinately regulated all iron uptake systems in E. coli, the fur (ferric uptake regulation) gene (Hantke, 1982). The fur gene product is thought to regulate the tonA, fhuB, fhuC, fecA, fecB, ent, fep, fes , tonB, cir, exbB, and the iron uptake region on ColV plasmids (Braun, 1983). Much of the knowledge about iron regulation has been determined with a technique developed by Casadaban to determine quantitatively the expression of genes involved in iron uptake (Casadaban & Cohen, 1979). Phage Mμ[Ap(ampicillin), lac(lactose)], which carries the structural genes for lactose utilization but lacks the regulatory elements of the lac operon, is integrated into a gene in the target host chromosome in the orientation of its transcription and thus the lactose structural genes come under the control of that gene. The amount of β-galactosidase produced was measured under iron sufficient and insufficient conditions. This technique has been used to determine that the tonA, fhuB, fepA, cir, tonB, exbB , and the iron uptake regions on the ColV plasmid are all regulated by iron concentration (Braun, 1983). There is insufficient information concerning the fec genes' regulation by iron since a mutant containing the Mμ phage inserted into one of the fec genes in the proper orientation for β-galactosidase production has not been obtained.

Aside from the more obvious changes in outer membrane protein profiles, another change has been found when E. coli is grown under iron stress in the presence of transferrin, lactoferrin, or ovatransferrin. Iron-replete cells have a t-RNA composition different than cells grown under iron stress. The t-RNAs for phenylalanine, tyrosine, tryptophan, and serine elute earlier on a column than do the same t-RNAs from cells grown under iron sufficiency (Buck & Griffiths, 1982; Griffiths et al., 1978; McLennan et al., 1981). These altered t-RNAs lack a methylthio(ms^2) moiety on an adenosine base found adjacent to the 3' end of the anticodon of the t-RNA. This alteration may have a regulatory effect since it has been found that the loss of the methylthio group reduces the translational efficiency of the molecule (Buck & Griffiths, 1982). Their evidence suggests that these altered t-RNAs relieve transcription termination at the attenuators of certain operons of the aromatic amino acid biosynthetic pathway and thus lead to the increased expression of these operons under iron limitation conditions. Enterochelin, the siderophore common to all enteric bacteria, is synthesized by way of a branch of the aromatic amino acid biosynthetic pathway (Rosenberg & Young, 1974) with iron being a part of the first enzyme of the common pathway (McCandliss & Herrmann, 1978). It is of interest to note that these changes in t-RNAs have also been found with E. coli growing in vivo under conditions of iron stress in the peritoneal cavities of lethally infected animals (Griffiths et. al., 1978).

The role of iron serving as a regulator is not new. Toxins, such as diphtheria toxin from Corynebacterium diphtheria (Pappenheimer & Johnson, 1936; Pope, 1932), toxin A from P. aeruginosa (Bjorn et al., 1978), shiga toxin from Shigella dysenteriae 1 (van Heyningen & Gladstone, 1953),

tetanus toxin from Clostridium tetani (Mueller & Miller, 1945), and alphatoxin from C. perfringens (Pappenheimer & Shaskan, 1944) are regulated by iron as well as other virulence factors such as elastase, alkaline protease, and hemagglutinin of P. aeruginosa (Bjorn et al., 1979). Even though the molecular mechanism of the iron regulation on these cellular products has not been elucidated, it is felt that the ability to grow under conditions of iron limitation as well as produce substances deleterious to the host has been most important in the evolution of pathogenic bacteria.

It is clear from the work of Klebba et al. (1982) that E. coli can detect and respond rapidly to fluctuations in extracellular iron availability. Using the natural biological chelator apotransferrin, ovatransferrin, and deferriferrichrome A to bind available medium iron, they examined the biosynthesis of six iron-regulated membrane proteins. The synthesis of five proteins with molecular weights of 83,000 d (83K), 81K (Fep), 78K (Ton A), 74K (Cir), and 25K were induced under iron deprivation conditions and their induction was also affected by the initial iron availability. That is, cells exposed to iron deprivation induced these proteins rapidly whereas iron-rich cells began induction only after a lag. A 90K protein's synthesis was inhibited by iron deprivation but stimulated by iron repletion and it was suggested by these authors that this protein may be used in iron storage.

The following set of sub-headings will examine in detail some of the better studied systems of bacterial iron sequestration:

1. Ferric enterobactin transport in Escherichia coli.

The structural genes for the seven biosynthetic enzymes (entA to entG) used to synthesize enterobactin (enterochelin) map near minute 13

on the *E. coli* chromosome. Enterobactin, formation constant for iron of 10^{52}, is a cyclic trimer of 2,3-dihydroxybenzoylserine that is formed from the precursors L-serine and 2,3-dihydroxybenzoic acid (DHBA), the latter being formed from chorismate. The gene products of *entA*, *entB*, and *entC* are involved in the formation of DHBA from chorismate (Young et al., 1971) while the products of genes *entD*, *entC*, *entF*, and *entG* make up enterobactin synthetase which converts DHBA and L-serine into enterobactin (Luke & Gibson, 1971; Woodrow et al., 1975). Cox et al. (1970) first described an *E. coli* mutant (*fep*) that was defective in ferric enterochelin uptake. The product of the *fepA* gene is an 81,000 d outer membrane protein (Fep A) which has been designated the ferric enterobactin receptor where initial binding of the ferric enterobactin to the cell envelope occurs (Hancock et al., 1976; Pugsley & Reeves, 1976). This protein also serves as the receptor for colicin B. Guterman and Luria (1969) were the first to show a relationship between colicin B and enterochelin with later studies demonstrating that enterochelin protects cells from colicin B activity by acting as a inhibitor of colicin adsorption (Guterman, 1973). There is now considerable evidence that the outer membrane receptor for ferric enterochelin uptake and for colicin B are the same (Hollifield & Neilands, 1978).

Other genes involved with the enterobactin iron transport system in *E. coli* are the product of the *fes* gene which codes for an esterase activity that enables release of the iron from the internalized ferric enterobactin complex (Langman et al., 1972), the *fepB* gene which has been postulated to be an inner membrane permease based on studies with *E. coli* spheroplasts (Wookey & Rosenberg, 1978), and two other membrane protein products those specified by the *tonB* and *exbB* genes which code for the

general membrane functions required for the transport of all iron compounds and vitamin B_{12} (Frost & Rosenberg, 1975). There has existed a controversy over the function and location of the tonB gene (Wookey & Rosenberg, 1978; Weaver & Konisky, 1980). Konisky (1979) and Weaver & Konisky (1980) have proposed that the tonB product serves as a physical link between inner and outer membrane components of these systems, an energy dependent 'gate', while Wookey & Rosenberg (1978) felt the tonB gene product was required for transport across the inner membrane. It is interesting that only those systems using outer membrane receptors have been found dependent on a functional tonB product. The gene order of the ent cluster on the E. coli chromosome is entD, fes, fepA, entF, fepB, entC, entA, entG, entB, and entE (Laird & Young, 1980; Pierce et al., 1983; McIntosh, 1983) starting from purE at 11 min and going toward the tonB at 27 min and exbB at 64 min.

Synthetic analogs of enterobactin were studied for their ability to support the growth of E. coli K-12 under iron limitation (Heidinger et al., 1983). The cyclic compound MECAM [1,3,5-N,N'N"-tris(2,3-dihydroxybenzoyl)-triaminomethylbenzene] and its methyl derivative promoted growth whereas sulfonated derivatives did not. However, linear sulfonated derivatives did reverse the growth inhibition. The uptake of active analogs required fepB, fesB, and tonB; morever, MECAM protected cells from colicin B.

2. Ferrichrome transport in Escherichia coli. The ferrichrome transport system in E. coli demonstrates a special case where E. coli has maintained a functioning transport system for a complex, ferrichrome, that it does not produce but is produced by all Penicillium molds and many other fungal species. This siderophore binds iron with a formation

constant of 10^{29}. As mentioned earlier, the tonA gene product was designated the ferrichrome receptor through studies demonstrating, for one, that ferrichrome was a potent specific inhibitor of plaque formation by the bacteriophage Ø80 (Wayne & Neilands, 1975). Moreover, the tonA gene product, 78,000 d protein on SDS-PAGE, has been shown to bind ferrichrome *in vitro* (Luckey et al., 1975). The tonA gene has also been designated the fhuA gene, ferric hydroxamate uptake, (Kadner et al., 1980); however, this designation has not been well received because it implies little ligand specificity whereas studies by Wayne (Wayne & Neilands, 1975) showed that the inhibition of phage plaques of Ø80 by ferrichrome was indeed very specific. Coulton et al. (1983) cloned a 3.5 kb DNA fragment from the *E. coli* chromosome that contained the fhuA gene. The Fhu A^+ phenotype was conferred on *E. coli* P8 which normally lacks the ferrichrome iron receptor protein of 78,000 d and a protein, the presumptive fhuA gene product of 78,000 d appeared in the outer membrane protein profile. *E. coli* tonA mutants are not only unable to sequester iron from ferrichrome but are resistant to phages T1, T5, Ø80, colicin M and the ferrichrome analog albomycin. Fecker & Braun (1983) cloned the fhu region into the plasmid pBR322. Using restriction endonuclease analysis, Tn5 insertion mutations, and complementation studies they found at least four genes involved in the transport of ferrichrome. These genes are transcribed in the following order: tonA (fhuA), fhuC, fhuD, and fhuB. The fhuCDB region is involved in iron supply to the cell from ferrichrome as well as three other hydroxamates, aerobactin, rhodotorulic acid, and coprogen. However, the outer membrane protein receptor for ferrichrome is the tonA gene product (Wayne & Neilands, 1975), for aerobactin the cloacin receptor protein (Iut) (Grewal et al., 1982), and

for ferric coprogen and rhodotorulic acid the Fhu E protein of 76,000 d (Hantke, 1983).

Mutations in the fhuCBD region imparts a phenotype that is unable to use any type of hydroxamate siderophore and the (fhu) designation seems more appropriate. It had mostly been believed that the fhuB gene product was an inner membrane protein since studies with pronase treated cells remained transport negative while a control tonA mutant acquired the capacity to transport ferrichrome under the same experimental conditions (Wookey et al., 1981); moreover, kinetic studies have shown iron-starved E. coli cells of an ent mutant possessed inner membrane vesicles capable of transport of (^{3}H) ferrichrome with a Km (Michaelis constant) of 0.2 μM (Negrin & Neiland, 1978) suggesting that there exists a discrete transport system in the inner membrane. Hider et al. (1980) found that ferrichrome transport is interrupted by metabolic inhibitors and there is some evidence to suggest a symport transport mechanism possibly involving divalent cations. However, through molecular cloning analysis, Prody & Neilands (1984) have recently presented evidence for an outer membrane location for the fhuB gene product which may be a 20,000 d protein. Furthermore, these authors found using radioactive iron uptake studies with ferrichrome and rhodotorulic acid, that in Kaback (inner membrane) vesicles both tonB and fhuB mutants transported the substrates in an energy dependent manner. Fecker & Braun (1983) suggested that the Fhu C and Fhu D proteins were cytoplasmic membrane proteins since they were solubilized by Triton X-100 in the presence of $MgCl_2$.

3. Ferric Citrate Transport in *Escherichia coli*.

Ferric citrate transport in *E. coli* is yet another way that this organism can sequester iron for growth. Based partially on the finding that citrate stimulates both growth and iron uptake in a *fep* mutant of *E. coli*, Cox et al. (1970) concluded that the organism possessed a citrate-mediated iron transport system. When *E. coli* cells were grown in the presence of citrate (formation constant for iron of 10^{25}), a substrate which *E. coli* cannot use as a carbon source, a citrate transport system was induced. The *fec* gene locus (ferric citrate) was determined by Woodrow et al. (1978) to be located at six minutes on the *E. coli* chromosome which is distinct from the *tonA* gene and genes of the enterochelin system. It is believed that the citrate system of iron uptake was genetically distinct from the enterochelin and ferrichrome systems since *fec* mutants did not affect iron sequestration by enterochelin or ferrichrome and conversely the *fep*, *fes*, *ent*, *tonA*, and *fhu* mutations did not affect iron uptake via citrate. Subsequently, the *fec* gene locus was divided into *fecA* and *fecB* with the *fecA* gene coding for the 80,500 d (SDS-Page analysis) (Wagegg & Braun, 1981) Fec A outer membrane protein (Hussein et al., 1981). This protein has been shown to be present in cells grown in 1 mM citrate and iron but to a much lesser extent in cells grown without citrate supplementation (Hancock et al., 1976). The presence of both iron and citrate are needed for the induction of the Fec A outer membrane protein. It is of interest that *Salmonella typhimurium* is able to use citrate as a carbon source but cannot utilize iron citrate (Pollack et al., 1970).

4. Ferric aerobactin transport in *Escherichia coli* (to be reviewed in greater detail in section D.1.)

Aerobactin is a citrate hydroxamate siderophore first isolated by Gibson & Magrath (1969) from *Aerobacter aerogenes* (*Enterobacter aerogenes*). A question has arisen as to the multiplicity of uptake systems that *E. coli* possesses, especially those strains that transport iron via the enterobactin system (formation constant for iron of 10^{52}) and the aerobactin system (formation constant for iron of 10^{23}). Neilands (1982) has suggested that iron is an element that *E. coli* has determined must never be eliminated from its dietary regime independent of its environment. Aerobactin has been purified from *Aerobacter aerogenes* (Gibson & Magrath, 1969), *E. coli* (Stuart et al., 1980), *Salmonella austin* (Warner, et al., 1981) *S. memphis* (Warner, et al. 1981), *Arizona hinshawii* (Warner et al., 1981), *E. cloacae* (vanTiel-Menkveld et al., 1982), *S. flexneri* (Payne, 1980) and some strains of *S. boydii* (Payne, 1983). Those *E. coli* that have been known to produce aerobactin have been shown to contain the genes for aerobactin synthesis on the ColV (colicin V) plasmid; however, work by Valvano and Crosa have recently shown that aerobactin is chromosomally encoded by certain clinical strains of *E. coli* (Valvano & Crosa, submitted for publication). Cloacin DF13, a bacteriocin produced by strains of *E. cloacae*, is lethal to nonimmune strains of *E. coli* and *E. cloacae*; however, vanTiel-Menkveld et al. (1981) found that ferric aerobactin protects these two organisms against cloacin DF13 and it is believed that this protection is due to a common receptor site in the cells' outer membrane. The ferric-aerobactin receptor, or at least a protein part of the ferric aerobactin receptor, has been determined to be a 74,000 d

outer membrane protein by SDS-PAGE analysis (Grewal et al., 1982). As mentioned in the preceding section, the fhuB, fhuC, and fhuD genes were required for uptake of ferric aerobactin (Fecker & Braun, 1983) and it should also be mentioned that the tonB, and exbB were required (Braun et al., 1982).

Konopka et al., (1982) demonstrated that both aerobactin and enterobactin were capable of removing iron (III) from transferrin in a buffered solution; however, aerobactin displaced the iron much more slowly than enterobactin with the rate for aerobactin mediated removal accelerated by pyrophosphate. These authors suggested that the transfer of iron (III) from transferrin to aerobactin may proceed through a ternary complex involving iron transferrin and aerobactin. They also found that not only the affinity constants but also the chemical structure of the competing ligand play an important role in the rate of iron mobilization from transferrin. Despite the superior affinities for iron of deferriferrichrome A and deferriferrioxamine B (two hydroxamate chelators) compared to aerobactin, they show a significantly slower rate of iron removal from transferrin than does aerobactin. However, this rate can be changed by addition of certain anions to the reaction mixture (Pollack et al., 1977). In the presence of PPi (pyrophosphate), Konopka et al. (1982) suggested the iron (III)-transferrin is converted to a apotransferrin-like conformation (Bates, 1981) and the labilized Fe (III) is released and forms a stable complex with aerobactin. In serum, the measured rate of aerobactin removal of iron (III) from transferrin was greater than for enterobactin especially when receptor-bearing cells of *E. coli* were present to act as a 'sink' for the iron. Thus, aerobactin must be endowed with special structural features favoring its adoption as

a plasmid specified iron carrier in clinical isolates of E. coli. Section D.1. discusses more on the aerobactin system of iron uptake with respect to its plasmid location.

5. Iron Uptake by Neisseria meningitidis and Neisseria gonorrhoeae

It had been shown that iron availability influences the virulence of Neisseria gonorrhoeae and N. meningitidis (Payne & Finkelstein, 1975; 1977; 1978). Using the test organism Arthrobacter flavescens J6-9 that has an absolute growth requirement for hydroxamic acids or hemin (Burnham & Neilands, 1961; Byers et al., 1967), halos of Arthrobacter growth formed around the colonies of N. gonorrhoeae and N. meningitidis indicating that these organisms produced a hydroxamate-class siderophore (Yancey & Finkelstein, 1981). As expected, the synthesis of gonobactin (the siderophore of the gonococcus) and meningobactin (the siderophore of the meningococci) was enhanced when the content of available iron was decreased. Meningobactin and gonobactin were easily dialyzable, poorly extractable in ethyl acetate and chloroform; however, they were extractable in chloroform-phenol as was the dihydroxamate siderophore schizokinen (Mullis et al., 1971). The piliated, virulent T1 gonococci produced more gonobactin than did the nonpiliated T3 strains consistent with the finding that the T1 gonococci are more virulent than the avirulent T3 gonococci and better able to acquire iron in the chicken embryo model (Payne & Finkelstein, 1975). Added gonobactin was also found to enhance the virulence of the gonococcus in chicken embryos (Finkelstein & Yancey, 1981). The meningococci produced three to five times more siderophore than the gonococci and both siderophore preparations could cross-feed the other culture but the homologous activity was more pronounced. These authors suggest that the compounds,

although similar, were not identical (Yancey & Finkelstein, 1981). There has been much debate in the past several years as to the mechanism whereby Neisseria obtain iron. The work from Dr. Finkelstein's laboratory has been very suggestive of a diffusible hydroxamate siderophore produced by the gonococci and meningococci; however, other workers have not found evidence for the classic E. coli like diffusible siderophores (Archibald & DeVoe, 1979; Simonson et al., 1982). Currently the detection of these siderophores is even more questionable due to the complication that a siderophore-like factor has recently been isolated after processing large quantities of control culture medium (R.A. Finkelstein, personal communication via J.H. Crosa). Archibald & DeVoe (1979) found that transferrin separated from a meningococcal culture by a dialysis membrane (12,000 d exclusion limit) did not permit growth of the organisms but transferrin added directly to the culture medium, so that cell contact with transferrin was possible, supported meningococcal growth. With the further studies by Simonson & DeVoe (1982) showing that culture supernatant fluids from the iron-deprived meningococci were unable to labilize ^{59}Fe from Sepharose immobilized transferrin, it was concluded that direct contact of the transferrin at the bacterial surface was necessary for the incorporation of transferrin iron. Kinetic studies used to elucidate the mechanism of iron release from transferrin for use by the meningococci were conducted using ^{59}Fe-transferrin, apotransferrin and bovine serum albumin. The purpose of these experiments was to determine if iron was recognized by an iron-specific cell associated siderophore or if the organism may recognize and even bind transferrin on its surface and then remove the iron via an iron reductase or a surface-attached siderophore (Simonson & DeVoe, 1983). The results

demonstrated that addition of apotransferrin significantly inhibited the acquisition of iron from ^{59}Fe-transferrin whereas bovine serum albumin, a protein of similar molecular weight, had no effect. This led the authors to conclude that the removal of iron must involve recognition of the transferrin molecule as an initial step in iron uptake. Since the transferrin molecule is about 80,000 d; its recognition by the bacterial cell must be almost certainly at the level of the bacterial outer membrane. Moreover, treatment of the cells with trypsin and also heating the cells for five min at 60°C eliminates their ability to use transferrin iron. Comparison of outer membrane protein profiles of the meningococci and the nonpathogenic neisseria N. flava, both under iron replete and iron deficient conditions, showed the appearance of a 69,000 d protein in the outer membrane profile of meningococci harvested under iron deficient conditions but on none of the other conditions or strains mentioned. This protein is a candidate for the transferrin receptor or the protein component of the transferrin receptor.

6. Miscellaneous studies of iron uptake by bacteria.

The Pseudomonas organisms are known to form several different structural types of siderophores: pyoverdine, pyochelin, pseudobactin, ferribactin, and various ferrioxamines. Moreover, they are able to use ferric citrate. Pyochelin from P. aeruginosa, a salicylic acid-substituted cysteinyl peptide (Cox et al., 1981), enables iron uptake in a two stage process (1) an energy-independent step (presumably binding to the cell surface) and (2) an energy-dependent process, accumulation of iron (Cox, 1980b). Only a single rate of uptake was observed by Cox (1980b) when cells were mixed with ^{55}Fe-citrate. Moreover, ferric citrate uptake which did not correlate with pyochelin

synthesis and was not induced by citrate, did involve an energy-dependent process. Iron uptake via the pyochelin system appeared to be controlled by the iron concentration in the growth media with the system most productive in low iron concentration. Sokol & Woods (1983) reported that outer membranes prepared from P. aeruginosa grown under low iron conditions bound more ferric pyochelin than membranes from cells grown under iron replete conditions. A protein of 14,000 d was found to bind ^{59}Fe-pyochelin. Pseudobactin was isolated from the fluorescent Pseudomonas B10 (Teintze et al., 1982) and characterized to be a linear hexapeptide. Moores et al. (1984), working with Pseudomonas B10, determined a minimum of 12 genes (arranged in four gene clusters) were required for biosynthesis of pseudobactin. The yellow-green, fluorescent, water-soluble pigment of P. fluorescens has been characterized and determined to meet the properties of a siderophore (Neilands, 1973): (1) specific derepression under conditions of Fe^{3+} deficiency and (2) very high affinity for Fe^{3+} (affinity constant of about 10^{32} for this fluorescent pigment) and (3) a lack of affinity for Fe^{2+} (Meyer & Abdallah, 1978). Studies demonstrating the role of pyoverdine in Fe^{3+} transport have been successful (Meyer & Hornsberger, 1978).

Russell & Holmes (1983) have reported on a dialyzable factor present in low-iron conditioned medium that was not present in high-iron conditioned media from growth of Corynebacteria diphtheria. This factor stimulated iron uptake by C. diphtheria and may be a candidate for a siderophore; however, the authors were not able to demonstrate, by chemical assay, the presence of phenolate or hydroxamate compounds in low-iron conditioned media. This iron uptake system was inhibited by the

addition of energy inhibitors as well as sulfhydryl group inhibitors. There are two types of iron-binding compounds in the *Mycobacteria*, the mycobactins, affinity constant > 10^{30}, (cell associated) (Snow, 1970) and the exochelins (extracellular) (Macham et al., 1975). Barclay & Ratledge (1983) proposed that the exochelins represent the most important means of iron acquisition for *in vivo* or *in vitro* growth of *Mycobacteria* while the mycobactins may serve as an intracellular iron storage compound. The siderophore, 2,3-dihydroxybenzoic acid (DHBA), is produced by *Bacillus subtilis* (Downer et al., 1970). Studies on the iron uptake system in *B. subtilis* have involved the mechanism of release of iron from the ferric chelate of DHBA. Siderophore reductase activity was found in cytoplasmic fractions of *B. subtilis* that was capable of catalyzing the reductive release of iron from ferri-DHBA (Gaines et al., 1981). The dihydroxamate siderophore schizokinen is secreted by the cyanobacteria *Anabaena* sp. strain 6411 and is a significant part of an iron uptake system (Lammers & Sanders-Loehr, 1982; Goldman et al., 1983) in this blue-green algae that may allow this organism a growth advantage over eucaryotic algae (Bailey & Taub, 1980; Murphy et al., 1976). The ability of *S. typhimurium* to grow in human serum and to cause lethal infection of mice is absolutely dependent on the production of enterobactin (Yancey et al., 1979) or 2,3-dihydroxybenzoate (Hoseith & Stocker, 1981), an enterobactin precursor. Two spermidine (catechol-type) siderophores, agrobactin from *Agrobacterium tumefaciens* (Ong et al., 1979) and parabactin from *Paracoccus dinitrificans* (Peterson et al., 1979; Tait, 1975) have been detected at low iron growth conditions.

Vibriobactin, like agrobactin, contains three 2,3-dihydroxybenzoyl residues, but contains two residues of L-threonine per molecule with a

polyamine backbone of norspermidine (Griffiths et al., 1983) and is the siderophore produced by Vibrio cholerae under conditions of iron stress. SDS-PAGE analysis using a mutant defective in vibriobactin transport has shown a 76,000 d protein altered with respect to both size and charge when compared with the wild type outer membrane protein profile of low iron cultured cells. The authors believe this protein may be a candidate for the vibriobactin receptor or a part of the receptor complex (Griffiths et al., 1983). A group of interesting intestinal pathogens, the Shigella spp. produce disease from the surface of the intestine or following limited invasion (LaBrec et al., 1964; Finkelstein, 1973) as do certain vibrios. Thus Payne (1980) began an investigation into their iron transport mechanisms. Most Shigella and Vibrio species synthesize phenolate iron binding compounds; however, S. flexneri and V. vulnificus synthesize hydroxamate siderophores which have not been found in other Shigella or Vibrio species. S. flexneri produces aerobactin as determined by thin layer chromatography and nuclear magnetic resonance (Payne, 1980). These studies also reported that S. flexneri could not synthesize enterobactin as could other Shigella species; however, further studies have shown that in certain isolates enterobactin was made (Payne, 1983). Hybridization studies have now revealed that most if not all S. flexneri contain the genes for enterobactin synthesis but that these ent^{+} strains perhaps use a different type of regulation of these genes than do the ent^{-} phenotypes (Payne, 1983). Both chromosomal genes as well as plasmid genes appear to be needed for enterobactin biosynthesis in the S. flexneri ent^{+} strains (Payne, 1983). The next section discusses the 'plasmid connection' in iron sequestration in two very well studied systems.

D. Iron Uptake Systems Mediated By Plasmid DNA.

1. The pColV-K30 system in *Escherichia coli*.

In 1969 the trivial name, aerobactin, was suggested for the dihydroxamic acid, consisting of a conjugate of 6-(N-acetyl-N-hydroxyamino)-2-aminohexanoic acid and citric acid. This compound was isolated from cultures of *Aerobacter aerogenes* grown under iron-deficient conditions (Gibson & Magrath, 1969). A few years later in 1974 Smith (Smith, 1974), working on a project that would seem unrelated to the work of Gibson and Magrath until about 1980, found that certain invasive strains of *E. coli* (F120) that caused bacteremia in chickens were able to transfer this lethal character to nonpathogenic *E. coli* K12. This increased lethality was not associated with the production of a toxic activity but with greater ability to survive in blood and peritoneal fluids. Strain F120 possessed the transmissible colicin V (ColV) plasmid (Hutton & Goebel, 1961; MacFarren & Clowes, 1967) and Smith concluded from his work that because the lethal character transfer always involved the transfer of the colicin V activity that the plasmid controlled lethal character was closely associated, or identical with colicin V. ColV plasmids are a class of plasmids approximately 100 megadaltons (Md) in size that all code for the small antibacterial protein colicin V (Gratia, 1925, 1932). Subsequently, Smith & Huggins (1976, 1978) showed that the elimination of the ColV plasmid from ovine, human, bovine, and avian *E. coli* strains was always accompanied by a decrease in pathogenicity. Reintroduction of the ColV plasmid into cured derivatives by conjugation restored pathogenicity to its original level. Even though there were reports in the literature that colicin activity could be responsible for the lethal character of the ColV plasmid due to

this activity causing increased vascular permeability and an inflammatory response in the host (Ozanne et al., 1977), the subject was further investigated. Using Tn1 transposon mutagenesis, Quackenbush and Falkow (1979) demonstrated that ColV plasmids that contained the Tn1 transposon inserted into the ColV structural gene so that colicin synthesis was inhibited were still more virulent for mice than were ColV$^-$ strains indicating that colicin V was not essential to virulence enhancement. Independent investigators also found that colicin V was not necessary for virulence (Williams & Warner, 1980). Since a link between ColV plasmids and invasive strains of *E. coli* had been established and the lethal plasmid property was probably not the production of colicin V, the search for the possible mechanism was pursued. It had been noted that bacteremic strains as well as strains from other extraintestinal sites possessed the ColV plasmids whereas no ColV$^+$ strains were detected among *E. coli* isolated from feces (Minshew et al., 1978). Since it had been reported that the availability of iron in the body of an infected host was crucial in determining the ability of an invading bacterial species to proliferate in tissues and body fluids (Bullen et al., 1978; Weinberg, 1978), Williams investigated the role of the ColV plasmid with respect to iron uptake in bacteremic *E. coli* strains (Williams, 1979). Addition of excess iron with the inoculum of test cultures in an experimental infection showed that the possession of the ColV plasmid gave the bacterium no selective advantage in causing disease; however, without the addition of 20 mM ferric ammonium citrate the ColV$^+$ strains were virulent whereas the isogenic cured ColV$^-$ strains were not. Moreover, addition of 3μM transferrin to the bacterial cultures showed only the ColV$^+$ strains able to grow unlimited lending further evidence to the idea that ability

to use iron may be the lethal character associated with the ColV plasmid. It was known at this time that iron-deprived E. coli produced the iron-chelating agent enterochelin (Hancock et al., 1976; O'Brien & Gibson, 1970) so using the mutant entA, from strain AN1937, which had a lesion in one of the steps in enterochelin biosynthesis, Williams compared AN1937 strains carrying different ColV plasmids for their ability to grow without the presence of added citrate (AN1937 cells require citrate for growth). E. coli AN1937 $ColV^+$ strains no longer required the addition of citrate for growth. However, the product of the tonB gene was required for growth in these $ColV^+$ containing strains. Direct measurement of the uptake of $^{55}FeCl_3$ showed a higher rate in the entA $ColV^+$ strain than the Ent^+ strain or the entA strain. In addition, the presence of the ColV plasmid in an Ent^+ background did not significantly alter the level of enterochelin excreted into the external medium in response to iron stress nor could any detectable enterochelin be found in entA $ColV^+$ strains. This work of Williams opened the doors for the search of the ColV plasmid associated product that could enable the bacteria to acquire the above list of properties. There was a precedent for plasmid-mediated factors associated with virulence of bacteria to host animals (Elwell et al., 1980). Several characters possessed by enteropathogenic E. coli are controlled by transmissible plasmids such as enterotoxin production (Smith et al., 1968; Smith et al., 1971; Smith et al., 1972; Skerman et al., 1972), K99 antigen (Smith et al., 1972), alpha-haemolysin (Smith et al., 1967), and K88 antigen (Orskov et al., 1966). Work on the mechanism of Yersina virulence has found that a 70 kb class of plasmids was associated with virulence and Ca^{2+}- dependent in vitro growth at 37°C (Portnoy, et al., 1981). At 37°C Y. enterocolitica

and Y. pseudotuberculosis alter their outer membrane profiles by producing at least three new outer membrane proteins; however, this expression was only seen in media containing a low concentration of Ca^{2+}, an alkali metal (Portnoy et al., 1984). Thus, the possession of a plasmid associated with the metal, iron, and its uptake and involvement with virulence was not surprising but it was unique.

Hydroxylamine-nitrogen was detected in cell pellets of $ColV^{+}$ cultures and in supernatant fluids but was not detected in strains from which the plasmid had been eliminated (Stuart et al., 1980). Moreover, the production of the hydroxamate was inhibited when added iron or citrate was added to the medium. While some researchers were working on the chemical identity of the molecule that enables iron sequestration in these $ColV^{+}$ bacteremic strains of E. coli, others were continuing their search for the genetic physiologic character of this plasmid mediated iron uptake system. Using mutagenesis of E. coli LG1513, which is enterochelin deficient but can still sequester iron via the ColV plasmid, pColV-K30, clones unable to grow on media containing the iron chelator dipyridyl were presumed to be defective in the ColV plasmid-mediated iron uptake system (Williams et al., 1980). Since mutants were desired in the plasmid system the chromosomal mutants had to be eliminated. To accomplish this the ColV plasmid was transferred by conjugation to a nalidixic acid resistant derivative of AN1937, LG1417 and the transconjugants were screened for their ability to take up iron. These plasmid mutants fell into two classes on the basis of cross-feeding bioassay tests, thus defining two plasmid-specified functions for the uptake of iron (1) iuc mutants were able to be cross-fed by a strain containing a wild type plasmid and were thus postulated to lack an

extracellular chelating agent for iron (2) iut mutants were not able to be cross-fed by strains producing the extracellular chelator and were felt to be defective in some aspect of the transport of the iron-chelator complex, possibly a membrane protein (Williams et al., 1980). One year later Warner et al. (1981) published that the pColV-K30 mediated chelator was identical, on the basis of field desorption mass spectrometry, with aerobactin, the siderophore synthesized by A. aerogenes as characterized initially by Gibson and Magrath. This shows the relationship of Gibson and Magrath's work on aerobactin synthesis in A. aerogenes and the invasive virulent strains of E. coli.

Grewal et al., (1982), using Tn801 transposition mutagenesis to generate an iut pColV-K30 mutant plasmid as well as using the iut mutant LG1419 which carried a revertable (point) mutation in pColV-K30 (Williams et al., 1980), found these iut mutants failed to induce a 74,000 d iron stress inducible outer membrane protein whereas the wild type strains displayed this protein. As mentioned earlier, these iut mutants did produce and secrete aerobactin but were not able to grow under iron-limitation conditions and were not able to be cross-fed by supernatants that contained chelators from wild type strains. All of this work in E. coli was performed in cir mutants because the cir protein, which is the receptor for colicin Ia, has a molecular weight of 74,000 d. Grewal et al. (1982) concluded that since the 74,000 d protein was inducible by conditions of iron stress and these iut mutants were defective in the uptake of ^{55}Fe, that this protein represents at least part of the ferric-aerobactin receptor. Further supporting evidence has found a 74,000 d protein in E. cloacae to be the aerobactin receptor (Oudega et al., 1979; van Tiel-Menkveld et al., 1981). van

Tiel-Menkveld et al. (1981) found the outer membrane receptor for ferric-aerobactin in E. cloacae was also the receptor for cloacin DF13, a bacteriocin produced by E. cloacae. In addition, E. coli which was usually resistant to cloacin DF13 became susceptible when a wild type pColV-K30 plasmid was introduced. Aerobactin competitively inhibits the lethal effect of cloacin DF13; moreover, those cloacin resistant mutants of E. coli that harbor a ColV plasmid are found to lack a plasmid specified 74,000 d outer membrane protein. Recently, two different research groups cloned the genes responsible for the complete iron uptake system and for just aerobactin synthesis from the pColV-K30 plasmid (Bindereif et al., 1983), and the cloacin DF13/aerobactin receptor (Krone et al., 1983). This was the first demonstration that the genes coding for the synthesis of aerobactin and the receptor protein were actually encoded on the plasmid pColV-K30. Using HindIII, Bindereif digested to completion purified pColV-K30 DNA and ligated these fragments into the HindIII site of the vector pPlac (Thummel et al., 1981). Selection of the desired clones, those containing the iron uptake system of pColV-K30, was made using the ampicillin resistance of the vector and a negative selection was made using cloacin. That is, those clones expressing a functional ferri-aerobactin receptor will be sensitive to cloacin. One clone, pABN1, that had acquired cloacin sensitivity contained an 18.3 kb plasmid which contained the 16.3 kb HindIII fragment B of pColV-K30. A subclone of this pABN1 clone, pABN5, was found to carry a 6.7 kb HindIII fragment and coded only for the aerobactin biosynthetic genes. These clones were characterized in E. coli BN3040 Nal^r(entA cir) and it was found that BN3040 Nal^r(pABN1) and BN3040Nal^r(pColV-K30) both expressed a 74,000 d protein when the cells were grown under iron limitation that was

absent in the host strain BN3040 Nal^r. Cells containing pABN1 but not pABN5 were cloacin sensitive. Culture supernatants from cells containing pABN1 and pABN5 were able to cross-feed *E. coli* LG1522 carrying the ColV-K30 *iuc* plasmid thus suggesting these cells synthesize aerobactin. This was confirmed by chemical analysis of culture supernatants. Krone et al. (1983) cloned a restriction fragment from pColV-K30 into the vector pBR322. Minicells of *E. coli* DS410 containing this plasmid, pFS8, expressed two polypeptides with molecular masses of 74,000 d (the cloacin DF13/aerobactin receptor protein) and 50,000 d. Cells harboring pFS8 were not subject to regulation by iron as were the cells containing pABN1 constructed by Bindereif et al. (1983). The fragment cloned by Krone et al. (1983) contained a *Bam*HI fragment which was located within the 16.3 kb *Hind*III fragment B that Bindereif et al. (1983) cloned. To investigate whether cells harboring pFS8 were able to take up Fe^{3+}-aerobactin, pFS8 was transformed to *E. coli* AN299-23 (*ent*A strain deficient in uptake of Fe^{3+}-enterochelin and Fe^{3+}-citrate), a strain incapable of growth under iron limitation. The results showed that this strain was able to take up iron into the cell as iron-aerobactin independent of the iron content of the medium; however, it was not able to grow under conditions of iron stress indicating that other genes may be involved in the release of iron from ferri-aerobactin into the cytoplasm. Five chromosomal genes, *fhu*BCD, *ton*B, and *exb*B were required for transport of iron (III)-aerobactin in ColV containing strains (Braun et al., 1982). Ferrichrome uptake in *E. coli* also uses these five chromosomal genes. Even though the mode of action of the *ton*B protein is unknown, it is known that the *ton*B function is essential for all iron

transport systems in E. coli and Salmonella sp. in which outer membrane receptor proteins participate.

The work I have just described was done using the E. coli isolates that contain plasmids that code for iron uptake; such as, pColV-K30, pRJ100, or pColV-K311. However, other types of ColV plasmids are known to enable bacterial virulence. Besides iron uptake virulence enhancing properties, the ColV plasmids pColV-IK94, pColV-F54, and pColV-F70 have all been found to encode for serum resistance (Aguero & Cabello, 1983; Nilius & Savage, 1984). Another pColV from E. coli K-12 strain 104-9A (Clancy & Savage, 1981) has been shown to code for adherence to epithelial cells.

2. The pJM1 system in Vibrio anguillarum.

Concurrent with the research work on the virulence plasmid pColV-K30, Crosa et al. (1977) reported that a plasmid was involved in the virulence of the fish pathogen Vibrio anguillarum. Working on the DNA relationships among marine vibrios pathogenic to fish (Schiewe et al., 1977), it was found that those pathogens contained a class of plasmids about 50 Md in size. It was this class of plasmids that Crosa began to study to determine if their presence could be correlated with the virulence of the organism. This plasmid class was not present in the low-virulent strains. DNA-DNA hybridization studies showed that all 50 Md plasmids were highly related independent of the geographic source which ranged from Japan to Maine, to Alaska, to Canada, to Washington, and Oregon. To study the correlation of virulence and plasmid carriage, Crosa et al. (1980) began to focus their studies on V. anguillarum strain 775 which contains only one plasmid, pJM1, a 47 Md (earlier referred to as the 50 Md class) plasmid. They were able to "tag" the pJM1 virulence

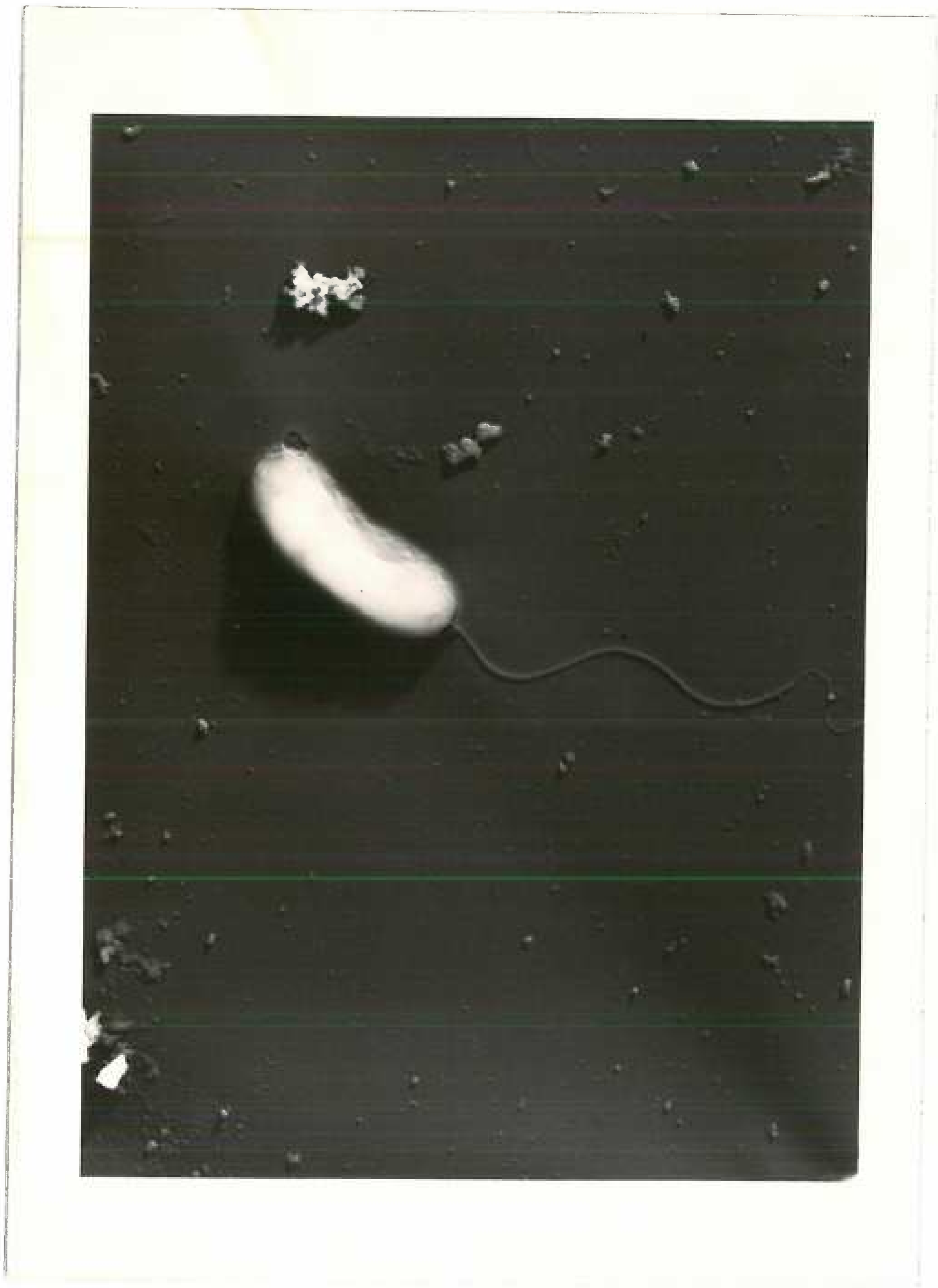
plasmid with the ampicillin resistance transposon A sequence Tn1 (Hedges et al., 1974) from the E. coli R plasmid RP4 (Datta et al., 1971; Grinsted et al., 1972). This genetic manipulation made it possible to follow the loss of the pJM1 plasmid in curing experiments which were used to confirm the relationship in isogenic strains between plasmid carriage and virulence. It had been the investigators experience that continued passage of virulent V. anguillarum 775 (pJM1) cultures at temperatures higher than their optimal growth temperature could attenuate virulence. Using a Tn1 derivative strain V. anguillarum 775 (pJM11), which was virulent in the fish model system, these investigators found that curing of the pJM11 plasmid (followed by loss of ampicillin resistance) by either heat treatment or chemical treatment with ethidium bromide was associated with an attenuation of virulence (LD_{50}) of about 3 logarithms. Because V. anguillarum 775 caused a septicemia and thus must be able to obtain iron in the uninviting environment of the host blood stream, Crosa investigated the possibility that V. anguillarum 775(pJM1) may possess a plasmid-mediated iron sequestering mechanism (Crosa, 1980). Using the plasmid-containing, high virulence 775(pJM11) and its low virulence heat cured derivative H775-3, it was found that addition of transferrin at 2.3 μM inhibited the growth of the low virulence plasmidless H775-3 strain. This growth inhibition was totally reversed in the presence of excess iron as $FeCl_3$. The kinetics of radioactive (^{55}Fe) uptake of nongrowing cells of V. anguillarum under conditions of iron stress was evaluated using the plasmid containing 775(pJM1) and the plasmidless isogenic derivative H775-3 (Crosa et al., 1981). Under the same uptake conditions, nongrowing cells of the plasmid-carrying, high virulence V. anguillarum 775(pJM1) took up radioactive iron more rapidly than H775-3.

Moreover, uptake of (^{55}Fe) by 775(pJM1) was inhibited by the respiratory inhibitor KCN suggesting that the process must be uptake rather than simply binding to the bacterial membranes which is energy-independent (Cox, 1980). Physiologic studies on the survivability of V. anguillarum 775 showed that this organism was serum resistant probably by causing an interference with the hosts alternate complement pathway for bacterial defense (Trust et al., 1981). This trait was not correlated with the virulence plasmid.

To further study this plasmid-mediated iron uptake system SDS-PAGE analysis of total and outer membrane profiles were performed comparing 775 (pJM1) and H775-3 (Crosa et al., 1981; Crosa, 1981). Two outer membrane proteins were found to be induced under conditions of iron limitation in 775(pJM1); however, one outer membrane protein designated OM2 (86,000 d) was not present in the plasmidless avirulent H775-3 but the other inducible outer membrane protein designated OM3 (79,000 d) was present in H775-3. The OM2 low iron inducible outer membrane protein was plasmid associated and the OM3 low iron inducible outer membrane protein was believed to be chromosomally encoded. Using a plasmid copy number determination it was found that the pJM1 virulence plasmid copy number did not change as a function of the iron concentration in the growth medium. Once it had been determined that the pJM1 virulence plasmid mediated an iron uptake system, further characterizations from a genetic and physiologic approach were undertaken and are covered in this thesis in paper 1: Iron uptake system mediated by Vibrio anguillarum plasmid pJM1 (Walter et al., 1983), paper 2: Lack of homology between the iron transport regions of two virulence-linked bacterial plasmids (Walter et

al., 1984), Appendix A: Siderophore activity by Members of the Genus *Vibrio*, and Appendix B: DNA Sequencing of Regions in the pJM1 Plasmid.

Figure 1. The flagellated comma shaped bacterium *Vibrio anguillarum*.



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III. MANUSCRIPTS

PAPER 1.

The Iron-Uptake System Mediated by the

Vibrio anguillarum Plasmid pJM1

ABSTRACT

Plasmid pJM1 from an invasive strain of *Vibrio anguillarum* mediates an iron sequestering system that is associated with the ability of this bacterium to cause septicemia in marine fishes. This plasmid-mediated iron-uptake system was analyzed by using mutations caused by transposon Tn*1*. Restriction endonuclease analysis of iron-uptake deficient and proficient derivatives generated by insertion of Tn*1* and molecular cloning experiments have permitted us to localize the plasmid regions involved in the process of iron-sequestration to a stretch of about 20 kilobase pairs. In addition, the existence of two plasmid-mediated components involved in the process of iron-uptake in *V. anguillarum* was defined: a diffusible substance which functions as a siderophore and a nondiffusible receptor for complexes of iron-siderophore which we have tentatively identified as the pJM1 plasmid-mediated outer membrane protein OM2 of *V. anguillarum*.

INTRODUCTION

Vibrio anguillarum 775 is an invasive pathogen of marine fish which causes a terminal hemorrhagic septicemia. This prevalent marine pathogen has been particularly devastating in the marine culture of salmonid fishes (3,13,16,22). It causes the characteristic vibriosis bloody lesions in the musculature, hemorrhaging at the base of the fins, with internal inflammation continuing into a generalized septicemia; death ensues from hypoxia and dysfunction of various organs (16, D.P. Ransom, C.N. Lannan, J.S. Rohovec and J.L. Fryer, in press). Crosa et al.(9) found a relationship between the presence of the pJM1 plasmid in *V. anguillarum* and an enhanced capacity to produce overt disease. Curing of the pJM1 plasmid was correlated with a decreased virulence (7). This virulence plasmid specifies a very efficient iron uptake system (4,5) which is required for the bacteria to be able to utilize the otherwise unavailable host iron which is bound by high affinity iron binding proteins like transferrin in serum or lactoferrin in secretions (2,19,20,23). Concomitant with an efficient plasmid-mediated uptake of iron into the cell cytosol during growth under conditions of iron limitation (5,6), the synthesis of two novel outer membrane proteins is induced: OM2, an 86,000 dalton protein associated with the presence of the pJM1 plasmid and OM3, a 79,000 dalton protein of chromosomal origin (6).

In this paper, we describe Tn*1* transposon (12) induced mutations in the pJM1 iron uptake system. By using a combination of restriction

endonuclease cleavage analysis and molecular cloning we were able to construct a physical map of the pJM1 plasmid and localize a region involved in the production of essential components of the pJM1 iron-uptake system. Preliminary reports of these findings have been recently published (10,11).

MATERIALS AND METHODS

Bacterial strains. *V. anguillarum* strain 775 carrying the virulence plasmid pJM1 has been previously described (7,9). Tn*1* insertion derivatives of this strain are described in Table 1. *E. coli* K-12 J53 (RP4) Ap^r Km^r Nm^r Tc^r (resistance to ampicillin, kanamycin, neomycin, and tetracycline respectively) was used in conjugation experiments to transfer RP4 containing the transposition sequence Tn*1* (Ap^r) to *V. anguillarum* 775(pJM1) Rif^r, Str^r (chromosomal resistance to rifampicin and streptomycin respectively). *E. coli* K-12 RR1 F^-, *hsdS20*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5*, *mtl-1*, *supE44* was used in transformation experiments.

Conjugation and transposition experiments. Plasmid RP4 was conjugated from *E. coli* J53(RP4) to *V. anguillarum* 775(pJM1) by using a filter method. The donor and recipient cells were separately diluted into 10 ml of brain-heart-infusion broth (BHI) (supplemented with 1% NaCl in the case of *V. anguillarum*) and allowed to grow to log phase (10^8 cells/ml) at 37°C for the *E. coli* culture and 20°C for the *V. anguillarum* culture. A mixture containing 1.5 ml of each culture (1.5 x 10^8 cells of each strain) was passed through a membrane filter (0.2 μm pore diameter; Bio-Rad Laboratories, Richmond, California). The filter was then placed on Trypticase soy agar plates (TSA; BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% NaCl and incubated at 20°C for 6, 24 and 48 hours. After incubation, the filters were placed in 2 ml of BHI

broth supplemented with 1% NaCl and mixed gently to remove the cells. Samples of the mating mixtures were plated on TSA plus 1% NaCl plates containing ampicillin (1.5 mg/ml), streptomycin (20 μg/ml) and rifampicin (20 μg/ml) to select for V. anguillarum exconjugants. Each colony appearing on the selection plates was next transferred to two series of plates: one series was supplemented with ampicillin, streptomycin, and rifampicin as above and the other supplemented with kanamycin (1 mg/ml), streptomycin and rifampicin. Incubation was again carried out at 20°C for 48 hours. Ap^r Km^r and Ap^r Km^s exconjugants clones were obtained and stored at -70°C in BHI-broth plus 1% NaCl and containing 40% glycerol until further use. Ap^r Km^s exconjugants were likely to be transposition derivatives in which RP4 was no longer present (loss of kanamycin resistance due to loss of RP4 but retention of the Ap^r phenotype of the Tn1 sequence). Both types of exconjugants were now tested for ability to grow under conditions of iron limitation in M9 minimal medium (5) containing the nonassimilable iron chelator EDDA (ethylenediamine-di(o-hydroxyphenyl acetic acid)) at 10 μM and also analyzed for the presence of plasmid DNA. Clones were grown overnight in 3 ml of BHI broth supplemented with 1% NaCl and lysates were prepared by a quick lysis procedure (1). Determination of the presence of transposition sequences on plasmid DNA was carried out by electrophoresis in 0.7% agarose gels in Tris borate buffer (89 mM Tris base, 2.5 mM disodium EDTA (ethylenediaminetetraacetic acid), 89 mM boric acid, pH 8.2). Gels were electrophoresed, stained and photographed as previously described (9).

Bioassays for detection of siderophore activity. The ability of supernatant fluid to support the growth of iron-uptake deficient mutants

in iron-depleted medium was tested by impregnating a sterile disc with 10 μl of supernatant fluid from the growth of iron-proficient wild-type V. anguillarum 775(pJM1) strains or Tn1 derivatives that were still iron proficient. Strains were grown in M9 minimal medium containing 10 μM EDDA for 48 hours at 22°C. After centrifugation of the cells the supernatants were removed and filter sterilized. The discs were placed on a minimal agar plate containing 10 μM EDDA that had been seeded with a lawn of 0.1 ml (10^8 cells) of the iron-uptake deficient strain (either 775::Tn1-5, 775::Tn1-6, 775::Tn1-7, or H775-3). For the iron-uptake deficient mutants, which were grown in about 1 μM EDDA, the sterile filtered supernatant fluids were made to 10 μM EDDA before addition to the test disc.

Isolation of plasmid DNA. Large scale purification of plasmid DNA was performed by the methods of Hansen and Olsen (15) or Birnboim and Doly (1) modified as described (18). Further purification was achieved by two successive centrifugations in cesium chloride-ethidium bromide density gradients at 45,000 rpm for 16 hours at 15°C in the VTi 65 rotor in a Beckman ultracentrifuge L8-70. The rapid alkaline procedure of Birnboim and Doly (1) was used to screen for plasmid DNA in transposition and recombinant DNA experiments.

Restriction endonuclease cleavage analysis. Restriction endonucleases were used under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of cleaved DNA was performed in a horizontal 0.5% or 0.6% agarose gel using a Tris-borate buffer system (9). For mapping experiments restriction fragment bands were cut out of a 0.5% gel and placed into separate large dialysis bags. Electroelution of the DNA from the agarose into the

dialysis bag was carried out using a Tris-borate buffer (0.1 of the concentration specified above) for 3 h at 200 V (20 mA)(18). After reversing the current for 10 sec the dialysis bag contents were placed into Nalgene centrifuge tubes and agarose was sedimented by centrifugation at 17,000 x g at $4^{O}C$ for 10 min. The DNA-containing supernatant was made 0.3 M in sodium acetate and the DNA was precipitated by adding 2.5 volumes of absolute ethanol at $-20^{O}C$. Next the DNA was sedimented by centrifugation for 1 h at 29,000 x g at $-10^{O}C$. The precipitated DNA was washed with 70% ethanol ($-20^{O}C$) and the dried pellet resuspended in the desired volume of 6 mM Tris-HCl pH 7.5. DNA restriction fragments isolated in this manner were used in ligation experiments or for further treatments with other restriction endonucleases.

Molecular cloning into the plasmid vector pBR325. Partial and total restriction endonuclease cleaved pJM1 plasmid DNA was cloned into the cloning vector pBR325 (18). Phosphatase-treated vector was used in all the recombinant DNA experiments performed. New England Nuclear calf alkaline phosphatase was used according to the directions of the supplier. Ligation experiments were carried out at $15^{O}C$ for 24 h using a ratio of 2 pmoles of pBR325 phosphatase treated ends to 0.5 pmoles of *Bam*HI cleaved pJM1 plasmid DNA and 0.05 units of T4 DNA ligase (Bethesda Research Lab.; Gaithersburg, Maryland). Transformation of *E. coli* strain RR1 with the ligation mixture was carried out as described (8).

Analysis of membrane proteins. Total cell envelopes as well as outer membranes from *V. anguillarum* were prepared essentially as previously described (6). Bacterial cultures (10 ml) were grown overnight at $20^{O}C$ in M-9 minimal medium containing either 20 µM $FeCl_3$ (iron sufficiency

conditions) or EDDA at 10 μM for iron proficient derivatives or 2 μM for iron-uptake deficient mutants (iron limitation conditions). Cells were harvested by centrifugation and resuspended in 1.5 ml of 10 mM Tris-hydrochloride-0.3% NaCl (pH 8.0) and sonically treated. After a 60 sec centrifugation in a microcentrifuge (Eppendorf), the supernatant fluid, which contained the cell envelopes, was centrifuged for 1 hour at 37,000 x g in a Beckman J2-21 centrifuge. To prepare outer membranes, the cell envelopes were treated with 1.5% Sarkosyl (wt/vol, final concentration in 10 mM Tris-hydrochloride pH 8.0) at room temperature for 20 min to dissolve the inner membrane. Outer membrane material was collected by centrifugation at 37,000 x g for 1 hr and the preparation was washed twice with distilled water and stored at -20°C until ready to use. Pellets from total cell envelopes or outer membranes were resuspended in 50 μl of a buffer consisting of 62.5 mM Tris-hydrochloride(pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.001% bromphenol blue, and 5% beta-mercaptoethanol. Suspensions were boiled for 5 min and samples were applied to a 12.5% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 44:0.8) in 375 mM Tris-hydrochloride (pH 8.8) containing 0.2% SDS with a 3% stacking gel in 125 mM Tris-hydrochloride (pH 6.8) containing 0.1% SDS. The electrophoresis buffer was 2.5 mM Tris-base, 200 mM glycine, 0.1% SDS. After electrophoresis (100 V for 16 h), gels were stained with 0.05% Coomassie blue in 25% isopropanol-10% acetic acid. Gels were destained with 5% acetic acid and photographed.

Experimental infections. Virulence tests were carried out on juvenile coho salmon (*Oncorhynchus kisutch*), each weighing about 5 g. For each bacterial dilution tested, five fish were anesthetized with tricaine

methane sulfonate (0.1 g/liter) and inoculated subcutaneously at the posterior base of the dorsal fin with 0.1 ml of various dilutions of bacterial suspensions from a 24 h culture grown with shaking in M9 minimal medium plus glucose and Casamino acids (Difco) at 20°C. The range of cell concentrations tested was from 10^2 to 10^8 cells/ml. After bacterial challenge, test fish were maintained in fresh water at 12±1°C for at least 7 days. Mortalities were collected daily and the kidney material was examined by bacteriological culture techniques. Mortalities were considered to be due to *V. anguillarum* only when the bacterium was reisolated in pure culture. Virulence was expressed as LD_{50} (mean lethal dose) as determined by the method of Reed and Muench (21).

RESULTS

Transposition mutagenesis of the pJM1 plasmid. RP4 (12,14), a P1 incompatibility group plasmid, which possesses the transposition sequence Tn*1*, carrying ampicillin resistance determinants (17), was used to initiate a mutagenic analysis of the pJM1 plasmid iron-uptake region. We were able to obtain two classes of exconjugants: Ap^r Km^s and Ap^r Km^r . The phenotype Ap^r Km^s indicates that these exconjugants must have lost RP4 while retaining ampicillin resistance, suggesting that they must have been generated by Tn*1* transposition to either the pJM1 plasmid or the *V. anguillarum* chromosome. The Ap^r Km^r exconjugants must still contain RP4, although they could also have suffered a transposition event. Consequently, all of the exconjugants regardless of their phenotype were analyzed for their ability to grow under conditions of iron limitation by using minimal medium containing 10 μM EDDA. A series of clones were identified in which the molecular weight of the pJM1 plasmid (Fig.1 lane i) had been increased by about 4.9 kilobase pairs (kb), due to insertion of Tn*1* into the plasmid genome (Fig.1 lanes a through e, h, and j, and Table 1). In one of the strains, 775::Tn*1*-6(pJHC9-8) the plasmid has actually a deletion of approximately 19 kb, possibly induced by a Tn*1* integration and deletion process (Fig.1, lane k and Table 1). In other transposed derivatives insertions appear to be quite unstable and keep generating deleted plasmids after about fifty successive plate transfers (Fig.1, lane f and g). Most of the strains containing the pJM1-insertion derivatives were iron-proficient, indicating that insertion of Tn*1* took

place in a plasmid region non-essential for the iron-uptake process. However, the deletion mutant 775::Tn1-6(pJHC9-8) and two insertion derivatives 775::Tn1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) showed an impairment in their ability to grow under conditions of iron limitation (Table 1). Although not seen in Fig.1 (due to its low copy number) strains containing the pJM1::Tn1 insertion derivatives in lanes a through d and h also carried plasmid RP4 as determined by both the antibiotic resistance phenotypes and the restriction endonuclease cleavage patterns in Fig.3 (RP4 cleaved DNA can be more readily detected due to the increased fluorescence of the intercalated ethidium bromide which is higher than in covalently closed circular DNA). Presence of RP4 did not affect either the iron uptake properties or the virulence of *V. anguillarum* (Table 1).

Nature of the iron-uptake deficiency mutations. To investigate whether the iron-uptake deficient mutations could be assigned to a deficiency in siderophore activity or to a lesion resulting in either the absence of an active membrane receptor or a modification that renders it unable to recognize the iron-siderophore complex, cross-feeding experiments were performed. Table 1 shows that under conditions of iron limitation, the iron-uptake deficient mutants 775::Tn1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) were able to be cross-fed by supernatant fluids from strains carrying an intact pJM1 plasmid or Tn1 derivatives that were still iron proficient. Growth did not occur when the discs in the bioassay contained supernatant fluids from iron-uptake deficient mutants or from a plasmidless strain of *V. anguillarum*. This cross-feeding effect was specific for the aforementioned mutants, as wild-type plasmid-carrying strains could not cross-feed the deletion mutant 775::Tn1-6(pJHC9-8) or the plasmidless

H775-3 (Table 1). The ability to utilize the supernatant fluids from the growth of iron-limited cultures of wild-type plasmid-carrying strains suggests the presence of a siderophore-like substance in these supernatant fluids, which is absent in supernatant fluids from either the plasmidless strain, the deletion mutant 775::Tn1-6(pJHC9-8), or from two insertion derivatives 775::Tn1-5(pJHC-91) or 775::Tn1-7(pJHC-W7). The ability to utilize a diffusible siderophore produced only by wild-type plasmid-carrying strains indicates that the plasmid-mediated outer membrane receptors in the iron-uptake deficient mutants 775::Tn 1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) are still functional. Supernatant fluids from the deletion mutant 775::Tn1-6(pJHC9-8) could not cross-feed the insertion mutants 775::Tn1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) suggesting that the deletion process that generated the plasmid pJHC9-8 also affected the plasmid genes involved in siderophore biosynthesis or activity. This deletion mutant cannot be cross-fed by wild type plasmid-carrying strains indicating that the 19 kb deletion affected not only the putative siderophore genes or a regulatory region involved with siderophore activity but also the pJM1 plasmid genes for the cognate membrane receptor or a regulatory region involved with receptor activity.

Virulence and plasmid-mediated iron transport. The Tn1-generated iron-deficient mutants were no longer virulent. The LD_{50} of these mutants increased by approximately 10^4 (Table 1).

Analysis of membrane proteins. Analysis of the membrane proteins produced under iron limitation at 10 µM EDDA showed that the OM2 protein was induced in all the iron-uptake proficient insertion derivatives (Fig.2) but is not detected in those cultures grown in high iron concentration. Since the iron-uptake deficient transposition derivatives

could not grow at EDDA concentrations higher than 2 μM we used this concentration rather than 10 μM to investigate the induction of OM2 in these mutants. Fig.2, panels a and b, lanes C_2 and D_2, shows that the OM2 protein was induced in the iron-uptake deficient insertion mutants 775::Tn1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) respectively but was absent from the deletion mutant 775::Tn1-6(pJHC9-8) shown in lane B_2. In this case a novel protein, ΔOM2, of about 69,000 daltons was induced under condition of iron limitation. In all the iron-uptake proficient and iron-uptake deficient derivatives the chromosomally mediated OM3 protein was induced.

Construction of a physical map of the pJM1 plasmid. pJM1 DNA was cleaved by the BamHI restriction endonuclease into eight fragments of 19.9, 14.4, 10.9, 6.5, 4.9, 3.2, 2.7, and 2.1 kb (Fig.3 lane d) whereas SalI cleaved pJM1 DNA into four fragments of 29.7, 20.3, 13.2, and 1.4 kb (data not shown). In Fig.3 we also show the BamHI cleavage of the plasmid DNAs from the deletion derivative (lane c), and seven pJM1::Tn1 derivatives (lanes b, e, f, g, h, i, and j). The transposition sequence Tn1 possesses one site susceptible to BamHI at a position located at 1.4 kb from one of its ends, close to the gene for beta-lactamase. Tn1 is not cleaved by SalI. Hence, digestions with these two enzymes permitted us to locate the sites of Tn1 insertions on the plasmid DNA obtained from the iron-uptake proficient and deficient transposition derivatives. By performing double digestions with both BamHI and SalI on pJM1 DNA we were able to obtain a preliminary order of these restriction fragments. This order was confirmed by molecularly cloning into the pBR325 vector, partial digests obtained from cleavage of pJM1 with BamHI. By using a combination of these two methods with all the transposition derivatives,

we were able to obtain a physical map of the pJM1 plasmid with the sites of Tn1 insertions (Fig.4). Further studies with other restriction endonucleases (XhoI and KpnI) confirmed the assigned location and orientation of the Tn1 insertions (data not shown). In this figure the location of the deletion which generated pJHC9-8 is also shown. This plasmid is harbored by a strain that is iron-uptake deficient and can both be categorized in the bioassay to be siderophore and membrane receptor activity minus. Analysis of this deletion plasmid as well as those plasmids containing Tn1 insertions which generated iron-uptake deficient mutants which do not produce a functional siderophore but that have an operating membrane receptor allowed us to localize the iron uptake region of pJM1 within a 20 kb region of the plasmid which includes possibly all of BamHI fragment 1 and parts of BamHI fragments 5 and 6.

DISCUSSION

In recent years the virulence of invasive strains of E. coli causing bacteremias in humans and animals and of V. anguillarum causing septicemic disease in fish have been attributed to the presence of efficient plasmid-mediated iron-transport systems (4,5,24,25). In this paper we describe the molecular characterization of the pJM1 system by using the mutagenic capability of the transposition sequence Tn1. The R-plasmid RP4 was conjugated to V. anguillarum and used as the donor of Tn1, generating a series of iron-uptake proficient and deficient derivatives. By using a physiological bioassay we were able to classify the iron-uptake deficient mutants into two classes. Some mutants were able to be cross-fed by supernatant fluids from wild-type plasmid-carrying strains of V. anguillarum, and thus lack an active putative plasmid-mediated siderophore. Other mutants could not be cross-fed by supernatant fluids from wild-type plasmid-carrying strains and could not cross-feed members of the other class of iron-uptake deficient mutants. Mutants in this second class must have not only a lesion in genes involved in siderophore production or activity but also in DNA regions associated with the activity or biosynthesis of a membrane receptor. Plasmid DNA from mutants belonging to these two classes was analyzed by restriction endonuclease cleavage analysis and the sites of Tn1 insertions or deletions were located. The mutants behaving as siderophore-minus receptor-plus were identified as possessing insertions in the BamHI fragment 1 which is 19.9 kb. A deletion affecting portions

of this fragment resulted in a siderophore-minus receptor-minus phenotype. Analysis of the outer membrane proteins of the iron-uptake deficient mutants, 775::Tn1-5(pJHC-91) and 775::Tn1-7(pJHC-W7) which were able to be cross-fed indicated that these Tn1 insertions on the pJM1 plasmid which affected production of siderophore did not affect the receptor activity or the biosynthesis of the OM2 protein. The insertion-deletion process that generated pJHC9-8 from pJM1 both affected functional siderophore and receptor activity. In this case, concomitant with the loss of receptor activity there was a disappearance of the outer membrane protein OM2. Under conditions of iron limitation a novel protein of about 69,000 d was induced in this deletion derivative. We hypothesize that this novel protein, which we called ΔOM2, is a truncated OM2 protein, possibly generated by Tn1-mediated deletion of a portion of BamHI fragment 1 carrying the carboxy-terminal end of the gene for the OM2 protein. If so, OM2 would be the presumed plasmid-mediated outer membrane receptor for the iron-uptake system encoded by pJM1. Of course, this contention depends upon the demonstration that ΔOM2 is actually derived from OM2 and that the OM2 protein can specifically bind complexes of V. anguillarum siderophore with iron. Radioimmunological experiments to prove the relatedness between OM2 and ΔOM2 are in progress. To date we have been unable to generate, by Tn1 insertion mutagenesis, derivatives of a siderophore-plus receptor minus phenotype, although both siderophore-minus receptor-plus and siderophore-minus receptor minus phenotypes were obtained. Whether this is due to a regulatory problem, for instance existence of only one promoter for the genes for siderophore and receptor, awaits further analysis. Nonetheless, we have found that there is an intimate relation between plasmid-mediated iron transport and

bacterial virulence since any mutation leading to an iron-uptake deficient phenotype was also reflected in an attenuation of virulence. The construction of a physical map of pJM1 DNA, on the basis of specific cleavage of pJM1 DNA by BamHI and SalI restriction endonucleases and by molecular cloning techniques, allowed us to determine the location of the iron transport region on the pJM1 plasmid to about 20 kb of pJM1 DNA (Fig.4) which includes possibly the entire BamHI fragment 1 and parts of BamHI fragments 5 and 6. Further dissection and molecular cloning of restriction fragments within this region is currently being carried out.

Table 1. Tn1 insertion derivatives of *V. anguillarum*.

[a]Growth in minimal medium containing 10 μM EDDA was determined by measuring the optical density at 590 nm at 21, 48, and 72h.

[b]Cross-feeding experiments were carried out as described in the text. NA, not applicable; NT, not tested.

[c]Virulence was determined as LD_{50} (number of bacteria killing 50% of the fish inoculated by using the method of Reed and Muench (21).

Tn*1* insertion derivatives of *V. anguillarum*

Strain	Plasmid	Molecular size (kb)	Growth in 10 μM EDDA[a]	Able to[b]:		Virulence (LD_{50})[c]
				Cross-feed	Be cross-fed	
775	pJM1	65	+	+	NA	1.2×10^3
H775-3	Cured		−	−	−	2.1×10^7
775::Tn*1*-5	pJHC-91	70	−	−	+	7.0×10^8
775::Tn*1*-6	pJHC9-8	51	−	−	−	5.0×10^8
775::Tn*1*-7	pJHC-W7	70	−	−	+	9.0×10^8
775::Tn*1*-3	pJHC-W3	70	+	+	NA	2.2×10^3
775::Tn*1*-10	pJHC-W10	70	+	+	NA	1.0×10^3
775::Tn*1*-4	pJHC-W4	70	+	+	NA	1.5×10^3
775::Tn*1*-55	pJHC-W5	70	+	+	NA	0.9×10^3
775::Tn*1*-11	pJHC-W11	70	+	+	NA	1.0×10^3
H775-3	RP4	54.5	−	NT	NT	2.1×10^8

Figure 1. Agarose gel electrophoresis of plasmid DNA isolated from *V. anguillarum*. Plasmid DNA was prepared by an alkaline lysis procedure (1) and analyzed as described in the text. Lanes a through k, plasmid DNA: (a) pJHC-W3; (b) pJHC-W4; (c) pJHC-W5; (d) pJHC-W10; (e) pJHC-W11; (f) pJHC-W6, plate transferred; (g) pJHC-W7, plate transferred; (h) pJHC-W7; (i) pJM1; (j) pJHC-91; and (k) pJHC9-8. Lane 1, plasmidless strain H775-3. pl, plasmid DNA; chr, chromosomal DNA.

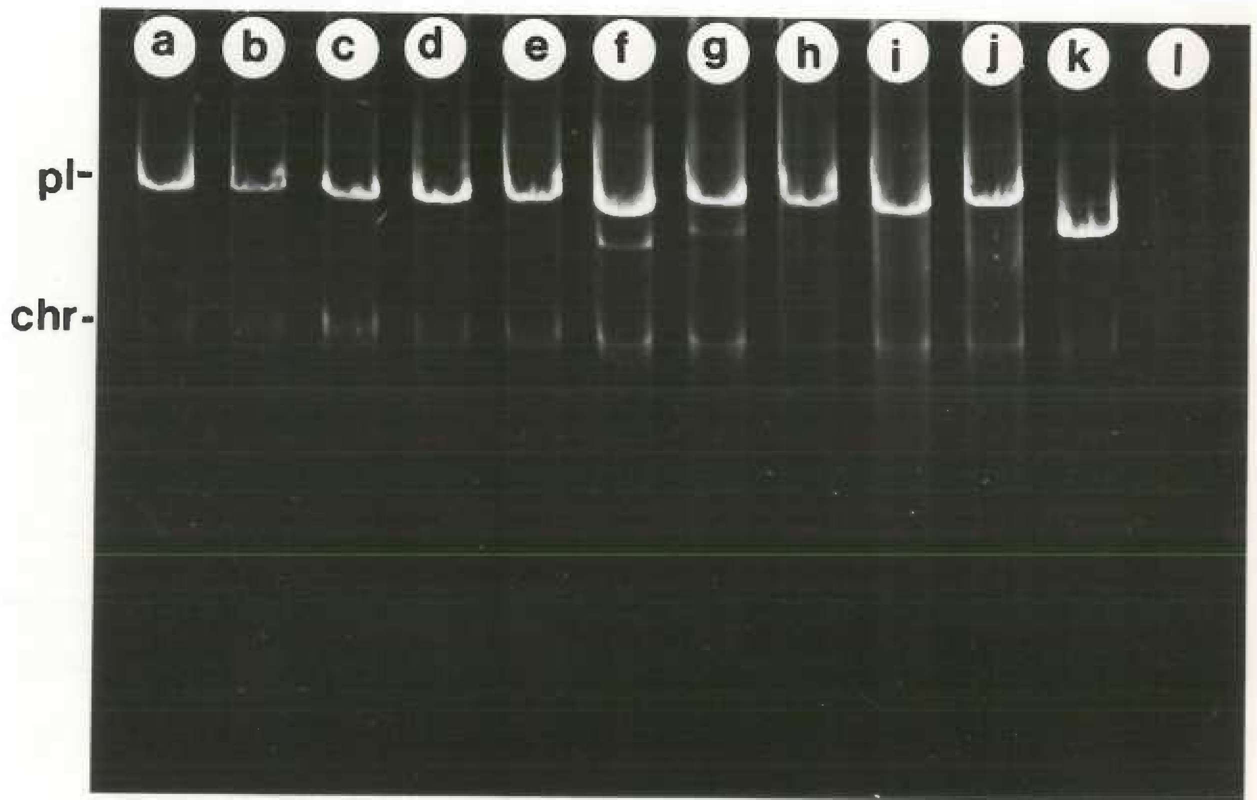
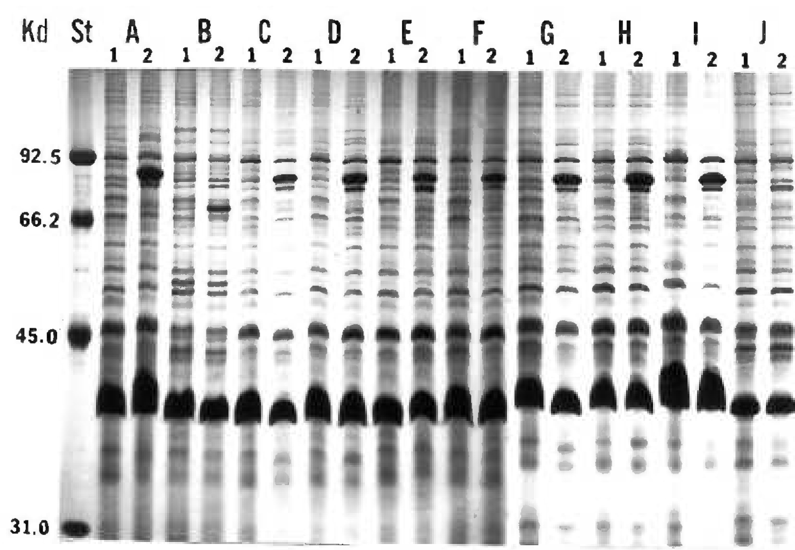

a
b
c
d
e
f
g
h
i
j
k
l
pl-
chr-

Figure 2. Total cell envelope (a) and outer membrane proteins (b) of Tn1 insertion derivatives of V. anguillarum . Kd, kilodaltons. Lane St, molecular weight standards; A, strain 775(pJM1); B, 775::Tn1-6(pJHC9-8); C, 775::Tn1-5(pJHC-91); D, 775::Tn1-7(pJHC-W7); E, 775::Tn1-3(pJHC-W3); F, 775::Tn 1-10(pJHC-W10); G, 775::Tn1-55(pJHC-W5); H, 775::Tn 1-4(pJHC-W4); I, 775::Tn1-11(pJHC-W11); J, plasmidless H775-3. Subscript 1 corresponds to membranes obtained from cells grown under iron sufficiency (20 μM $FeCl_3$) whereas subscript 2 corresponds to membranes from cells grown under iron limitation conditions (10 μM EDDA for iron uptake proficient derivatives and 2 μM EDDA for iron uptake deficient derivatives). The following symbols are used on panel b to identify specific outer membrane proteins: lane A_2, 2: OM2 protein; lane A_2, 3: OM3 protein; lane B_2, 3: OM3 protein, and lane B_2, *:ΔOM2 protein. The positions of the OM2 and OM3 proteins on the other lanes (C_2 through I_2) are identical to those shown in lane A_2. In lane J_2 (plasmidless strain) the only outer membrane protein induced under conditions of iron limitation is the chromosomally-mediated OM3.

a



b

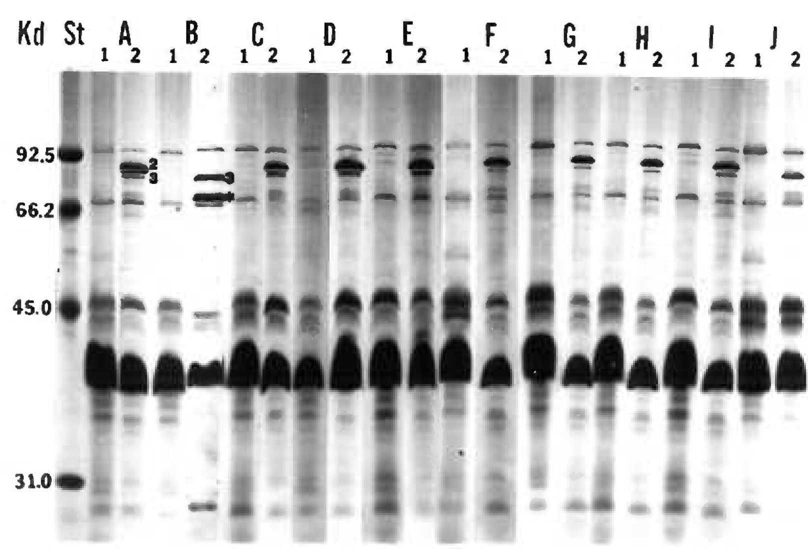


Figure 3. Restriction endonuclease cleavage analysis of *Tn1* insertion derivatives of the pJM1 plasmid. Restriction endonuclease reactions were carried out as described in the text. Lanes a and k: molecular weight markers (*Hind*III cleaved lambda DNA plus *Hae*III cleaved ØX174 DNA. Lanes b through j are *Bam*HI cleaved DNA from the plasmids: b: pJHC-W3; c: pJHC9-8; d: pJM1; e: pJHC-91; f: pJHC-W7; g: pJHC-W10; h: pJHC-W4; i: pJHC-W5; j: pJHC-W11. The top band in lanes b, f, g (light), h, and i is RP4 that has been cleaved at its only *Bam*HI site. kb: kilobase pairs.

a b c d e f g h i j k

kbp

23.5

9.7

6.6

4.3

2.2

2.1

1.3

1.1

0.9

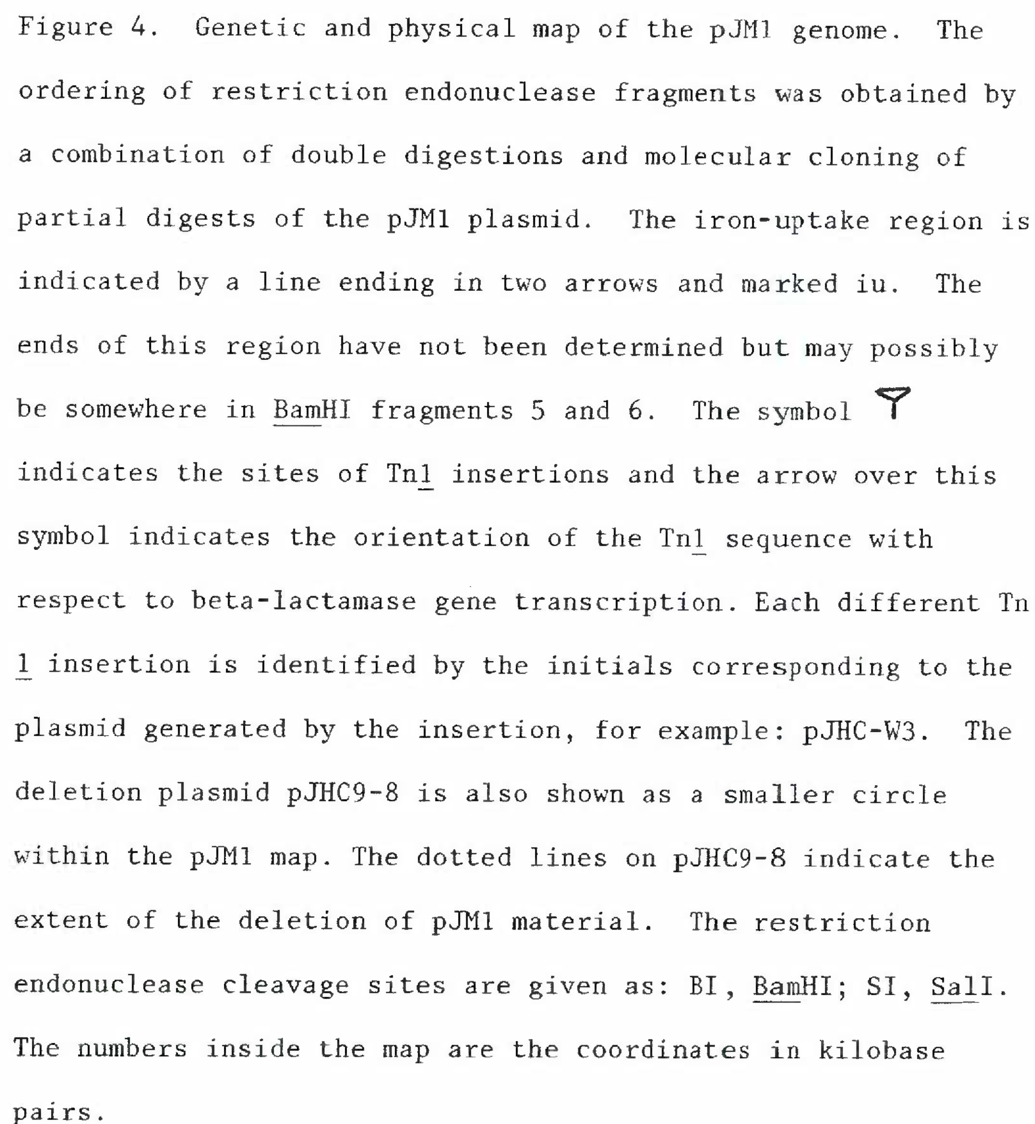
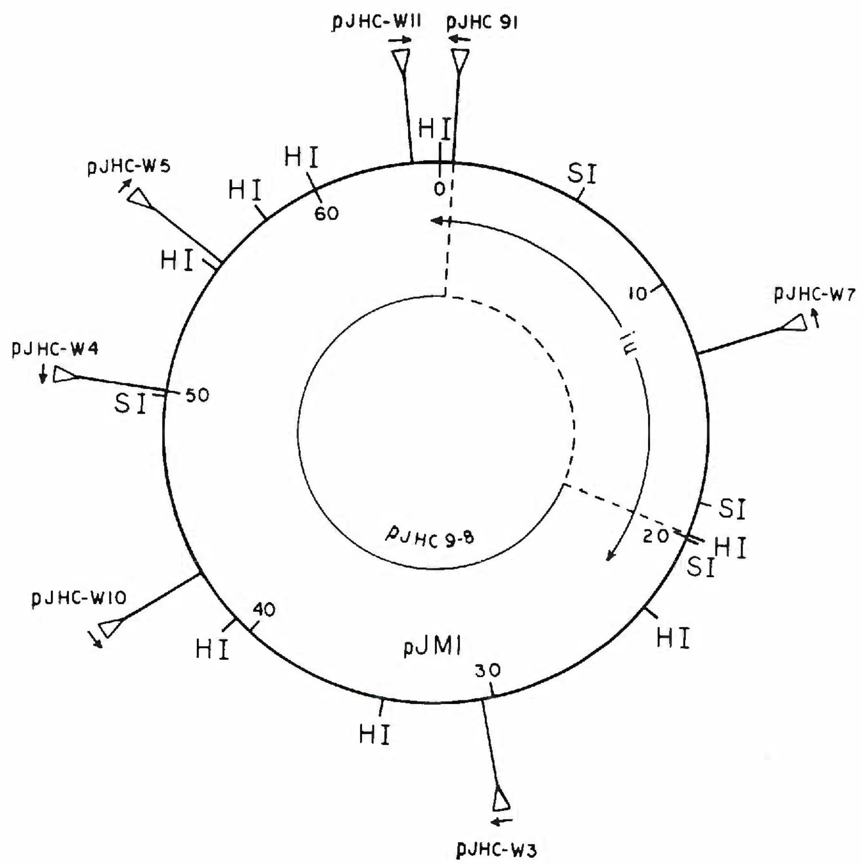


Figure 4. Genetic and physical map of the pJM1 genome. The ordering of restriction endonuclease fragments was obtained by a combination of double digestions and molecular cloning of partial digests of the pJM1 plasmid. The iron-uptake region is indicated by a line ending in two arrows and marked iu. The ends of this region have not been determined but may possibly be somewhere in *Bam*HI fragments 5 and 6. The symbol Y indicates the sites of Tn*1* insertions and the arrow over this symbol indicates the orientation of the Tn*1* sequence with respect to beta-lactamase gene transcription. Each different Tn *1* insertion is identified by the initials corresponding to the plasmid generated by the insertion, for example: pJHC-W3. The deletion plasmid pJHC9-8 is also shown as a smaller circle within the pJM1 map. The dotted lines on pJHC9-8 indicate the extent of the deletion of pJM1 material. The restriction endonuclease cleavage sites are given as: BI, *Bam*HI; SI, *Sal*I. The numbers inside the map are the coordinates in kilobase pairs.



pJHC-W11
pJHC 91
HI
0
SI
HI
60
HI
pJHC-W5
HI
10
pJHC-W7
pJHC-W4
SI
50
pJHC 9-8
SI
20
HI
SI
pJHC-W10
40
HI
HI
pJM1
30
HI
pJHC-W3

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PAPER 2.

Lack of Homology Between the Iron Transport Regions of Two Virulence-Linked Bacterial Plasmids

ABSTRACT

Two plasmids involved in bacterial virulence, the *Escherichia coli* plasmid pColV-K30 and the *Vibrio anguillarum* plasmid pJM1 have been studied with respect to the iron sequestering systems mediated by these two plasmids. Bioassay results show that the two systems are not related functionally because specific iron-uptake deficient mutants in each system cannot be cross-fed by the heterologous bacteria using culture supernatants from iron-proficient strains containing wild-type plasmids. DNA hybridization studies show an extensive lack of homology between regions involved in iron sequestration in both plasmids.

INTRODUCTION

In recent years the virulence of two types of bacteria belonging to different ecological niches was demonstrated to be due to the presence of specific plasmid classes (6,15). It was found that a significant proportion of *Escherichia coli* causing bacteremias in humans and domestic animals harbor a ColV plasmid. Cured strains showed decreased virulence in the mouse model (21). By using another vertebrate system, a salmonid fish, it was found that the virulence of highly pathogenic strains of the marine fish pathogen *Vibrio anguillarum* was correlated with the possession of the 47 Md plasmid pJM1. Curing of this plasmid caused an attenuation of virulence in this marine bacterium (5). Until recently it was not known what properties of these two plasmids were responsible for virulence, although it was obvious that the diseases caused both by *E. coli* and *V. anguillarum* are of a similar nature in the sense that they both are characterized by the establishment of a fulminant septicemia. Therefore, in considering the possible mechanisms by which these two organisms can cause disease, their ability to grow and be disseminated in the host vertebrate blood was investigated as a potential factor of virulence. One component that is essential for bacterial growth is iron. However, free iron is not readily available in the vertebrate host, being present mainly intracellularly (20). Circulating iron is bound by high affinity iron binding proteins, like transferrin in serum and lactoferrin in secretions (2a). These proteins are unsaturated in normal hosts and thus limit the availability of iron for any invading pathogens. Thus,

microorganisms have had to develop mechanisms whereby the otherwise unavailable iron could be assimilated by the invading bacteria. Recent work (3,4,21) demonstrated that the pColV-K30 and the pJM1 plasmids coded for novel iron transport systems which were highly efficient in obtaining iron from complexes of iron-transferrin. In vivo experiments corroborated the essential function coded for on these plasmids and thus these plasmid-mediated iron transport systems were demonstrated to be an important component of the virulence repertoire of these two bacteria. Because of the similar functions coded for by pJM1 and pColV-K30 plasmids in nonrelated bacterial species, we decided to investigate these two plasmid-mediated iron transport systems using both a functional and a molecular approach.

MATERIALS AND METHODS

Bacterial strains. *Vibrio anguillarum* 775 carrying the virulence plasmid pJM1 was previously described (5,6). *E. coli* K-12 294 carrying the plasmid pABN1 was previously described (2).

Bioassays for detection of siderophore activity. The ability of supernatants to support the growth of the *E. coli*(pColV-K30-1) and *V. anguillarum* (pJHC-91) iron-uptake deficient strains in iron depleted medium was tested by impregnating a sterile filter disc with 10 µl of supernatant from the growth of the wild type iron-proficient strains *E. coli* LG1315(pColV-K30) and *V. anguillarum* 775(pJM1). pColV-K30-1 (*iuc*), a derivative of pColV-K30, has a mutation in genes concerned with aerobactin synthesis (22), whereas pJHC-91 (*iuc*), a derivative of pJM1, has a mutation in genes concerned with the biosynthesis of the putative *V. anguillarum* siderophore. Supernatants were obtained from strains growing in M9 minimal medium (14) supplemented with glucose, casamino acids (Difco), and a non-assimilable iron chelator EDDA (ethylendiamine-di-o-hydroxyphenyl acetic acid) at 10 µM. After centrifugation of the cells the supernatants were removed and sterilized by filtration. Disks were added to bioassay plates containing 10 µM EDDA in minimal medium and 0.1 ml of an overnight culture of 10^8 cells per ml of the lawn strain of bacteria under iron stress (the *Iuc* mutants) and the desired 10 µl of sterile culture supernatant applied. A positive result for cross-feeding is represented as a halo of growth around the

applied disk. This bioassay is able to categorize strains as those that can be cross-fed and/or those able to cross-feed.

Preparation for hybridization analysis. Large-scale purification of plasmid DNA, restriction endonuclease cleavage analysis, and molecular cloning techniques have been previously described (18). Southern blot hybridization analysis of restriction endonuclease cleaved pColV-K30 and pJM1 DNA. Plasmid DNA was cleaved with either *Hind*III or *Bam*H1 restriction endonucleases (Bethesda Research Laboratories Inc.) and electrophoresed in two identical 0.6 % agarose gels in a Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid, pH 8.3) at 70 volts for 3 hours. The gels were removed, stained with ethidium bromide (1 μg/ml) and photographed. Restriction endonuclease cleaved plasmid DNA was next transferred from the gels to nitrocellulose filters (Schleicher and Schuell) by using the Southern blot technique (16). Nitrocellulose filters were next placed in plastic bags containing 5X SSC (1X SSC = .15 M NaCl, .015 M Na_3citrate), 50% formamide, Denhardt's solution (0.02% ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin (7)), 1mM EDTA, and 0.1% SDS (sodium dodecyl sulfate). After 3 hours at 37°C, the appropriate heat-denatured ^{32}P-labelled plasmid DNA (10^6 counts per min) was added and hybridization was carried out by incubation in the same preincubation solution with carrier salmon testes DNA at 100 μg/ml for 16 hrs at 37°C.

RESULTS

Iron-uptake deficient mutants were recently characterized in both the pColV-K30 and in the pJM1 systems by the use of a physiological bioassay, which defined the existence of at least two important components for iron transport (18,22). One is a diffusible substance which may play a role as a siderophore (12,13), and the other is a nondiffusible component which may be a membrane receptor for iron-siderophore complexes (10). In the case of the pColV-K30 system the siderophore was identified as the hydroxamate aerobactin (19) and the membrane receptor was presumed to be an outer membrane protein of 74,000 daltons (11).

To characterize the systems functionally, we decided to study the ability of supernatants from iron-limited cultures of *E. coli* carrying pColV-K30 and *V. anguillarum* harboring pJM1 to cross-feed iron-uptake deficient mutants of these bacteria characterized as being deficient in the production of the specific plasmid-mediated siderophore (*iuc*). In most experiments we used culture supernatants containing the plasmid-mediated siderophores although in some cases we also used purified aerobactin. Aerobactin could only cross-feed *iuc* mutants from the *E. coli* strain carrying the pColV-K30-1 plasmid, but it could not cross-feed *iuc* mutants of *V. anguillarum* containing the pJHC-91 plasmid (Table 1). Culture supernants from *V. anguillarum* cannot crossfeed the *E. coli iuc* mutant. Thus, it appears that from a functional standpoint the pJM1 and pColV-K30 plasmid-mediated iron transport systems are not related. In order to determine whether the two systems are related at the

plasmid DNA level, we decided to perform Southern blot hybridizations (16) of gels containing restriction endonuclease cleaved pColV-K30 and pJM1 DNA, using as radioactive probes plasmid clones carrying plasmid DNA regions involved in iron transport. Thus, we used the plasmid pABN1 (2) carrying the pColV-K30 iron-transport genes and the plasmid pJHC-W1 harboring pJM1 iron transport regions (18). The pColV-K30 iron uptake regions hybridized only with restriction endonuclease cleaved pColV-K30 DNA (Fig. 1A and B), whereas the clone containing pJM1 iron uptake regions hybridized only with restriction endonuclease cleaved pJM1 DNA and not with restriction endonuclease cleaved pColV-K30 DNA (Fig. 1C and D). These hybridization results clearly show that the iron transport regions of these two plasmids are not homologous under our assay conditions.

DISCUSSION

We should conclude from the experiments in this paper that there is no appreciable homology either functionally or at the DNA level between these two plasmid-mediated iron transport systems. From an evolutionary standpoint it is intriguing that both of these iron transport systems are required for virulence and are mediated by plasmids. Although it may appear that a lack of similarity in iron sequestration regions in plasmids carried by heterologous hosts is expected, there is precedence for interaction between heterologous systems at both a functional as well as a genetic level. For example, an important iron transport system in *E. coli* possesses an outer membrane receptor, the *ton*A protein, that can recognize a siderophore, ferrichrome, synthesized by the fungus *Ustilago sphaerogena*, which is not phylogenetically related to *E. coli*. Recent cross-feeding bioassay findings have shown that the addition of a *V. anguillarum* culture or supernatant can reverse the growth inhibition under iron limitation conditions of the human pathogen *V. fluvialis* suggesting some identity among iron uptake components (1). At the genetic level, *E. coli* is known to exchange genetic information by conjugation with other phylogenetically unrelated bacteria, like *Vibrio* sp.(9) including *V. anguillarum* 775, the strain used in this work (5). In addition, it was shown that conjugation can occur between the fish pathogen *V. anguillarum* and the human pathogen *V. parahaemolyticus* (8). Therefore, it is conceivable that the conjugative ColV plasmid or DNA

regions from this plasmid, such as the iron transport system, could be transferred to a V. anguillarum host. There is also precedence for the mobility of pJM1 and pColV sequences between genomes and bacteria. Our laboratory has recently demonstrated that certain virulent strains of V. anguillarum, which do not possess a pJM1-type plasmid, are iron-proficient and do possess chromosomal DNA sequences that are homologous to pJM1 plasmid DNA sequences (17). This mobility of plasmid DNA sequences is also found with the pColV plasmid iron regions since the genes for aerobactin synthesis have been found on plasmids from other enteric bacteria like Arizona hinshawii and Aerobacter aerogenes (S.McDougall and J.B.Neilands, Abstracts of the Annual Meeting of the American Soc. for Microbiol.-1983 p.208). Nevertheless, our results do not substantiate the presence of pColV iron transport regions in pJM1. At the DNA level, under the conditions used to perform our hybridization experiments, we would expect to detect even somewhat distant sequences. Certainly, our results do not preclude the existence of topological homologies at sites involved in regulatory events of the plasmid-mediated iron transport genes. This possibility awaits further dissection of both iron transport regions by DNA sequencing experiments which are currently being pursued.

Table 1. Bioassay with culture supernatants from plasmid-carrying, iron-proficient strains.

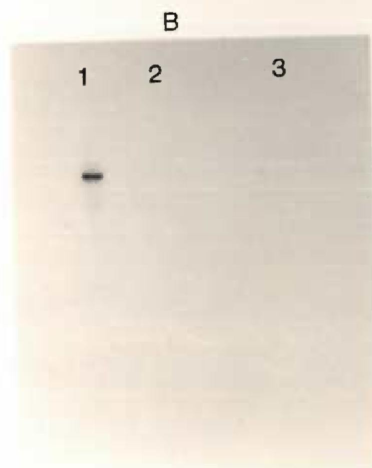
Bioassay with culture supernatants from plasmid-carrying, iron-proficient strains

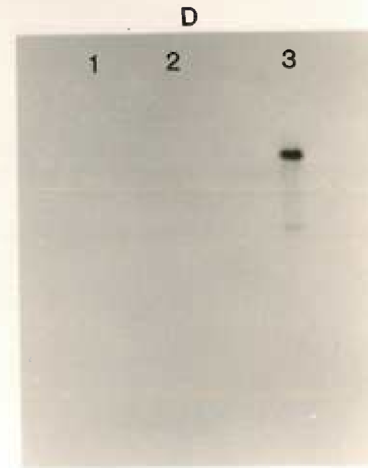
Strain used in lawns	Genotype	Plasmid	Cross-feeding with supernatants from:	
			E. coli LG1315	*V. anguillarum* 775
E. coli LG1522	*iuc*	pColV-K30-1	+	−
V. anguillarum 775::Tn*1*-5	*iuc*	pJHC-91	−	+

Figure 1. Southern blot hybridization analysis of restriction endonuclease-cleaved pColV-K30 and pJM1 DNA. *HindIII* cleaved pColV-K30 and *BamHI* cleaved pJM1 DNA were electrophoresed in 0.6% agarose gels, blotted to nitrocellulose filters, and hybridized against ^{32}P-pABN1 DNA or ^{32}P-pJHC-W1 DNA. Panels A and C are photographs of the duplicate 0.6 % agarose gels showing restriction endonuclease cleaved plasmid DNA. Lane 1: *HindIII* cleaved pColV-K30; Lane 2: *HindIII* cleaved unheated lambda DNA standard; Lane 3: *BamH1* cleaved pJM1 DNA. Panels B and D are photographs of the autoradiographs obtained when the above gel blots were hybridized with ^{32}P-labelled plasmid DNA. Panel B shows the results for the hybridization with ^{32}P-pABN1 DNA which carries pColV-K30 iron transport regions and panel D with ^{32}P-pJHC-W1, a clone carrying iron transport regions of pJM1.

A
1
2
3

C
1
2
3


B
1
2
3


D
1
2
3

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IV. APPENDIXES

APPENDIX A.

Siderophore Activity by Members of the Genus *Vibrio*

INTRODUCTION

Payne & Finkelstein (1978) found that Vibrio cholerae produced a phenolate-type siderophore that is similar to, but not identical to, enterochelin. More recently, Griffiths et al. (1983) characterized this siderophore, vibriobactin, as a catecholamide which does not possess spermidine as the polyamine backbone of the molecule. It has instead the rare N-(3-aminopropyl)-1,3-diaminopropane, norspermidine. This compound is produced under conditions of iron stress. Culture supernatants from such iron-limited cultures of V. cholera were able to reverse the growth inhibition of iron-starved cultures of V. cholerae, N. gonorrhoeae and N. meningitidis whereas enterobactin was not. Sigel & Payne (1982) found that at least six new outer membrane proteins were seen in SDS-PAGE gels when the cells were grown under iron stress conditions (220,000 d [220K], 77K, 76K, 75K, 73K, and 62K proteins). The 220,000 d protein was found in the cells at the same time as the appearance of siderophore activity. It appeared loosely associated with the outer membrane, being immunoprecipitated with antisera directed against the cell surface of iron-starved cells but was extracted by 0.5% Sarkosyl treatment (Filip et al., 1973). Of the five other outer membrane proteins, the 40K and 62K appeared to be peptidoglycan associated since they were solubilized only at 60°C or above (Rosenbusch, 1974; Lugtenberg & Alphen, 1983). Sigel & Payne (1982) suggested that the 40K protein was the major outer membrane protein and the 62K protein a porin [many E. coli peptidoglycan associated proteins are porins (Di Rienzo et al., 1978)]. Sciortino &

Finkelstein (1983) found that V. cholerae, grown in vivo in the infant rabbit model, showed a very similar outer membrane protein profile to in vitro cultures grown under iron stress, but were not similar to in vitro cultures grown in iron replete conditions. Moreover, in vivo preparations did exhibit unique proteins. Interestingly, when Sigel & Payne (1982) compared clinical V. cholerae isolates with an environmental isolate, differences in their siderophore production under iron limitation were found. Clinical isolates of V. cholerae, a non invasive pathogen that remains on the gut mucosal surface where the iron concentration may be high enough to repress the high affinity mechanism, produced less siderphore per unit of cell (as much as 4 times less) than did the environmental isolate. The environmental strain, isolated from brackish waters in Chesapeake Bay, continued to sequester iron by its high affinity system at iron levels that repressed expression in all of the clinical isolates.

Simpson & Oliver (1983) examined the siderophore production of the halophilic pathogen V. vulnificus. Supernatants from V. vulnificus low-iron cultures contained siderophore activity. Ethyl acetate extracted material supported the growth of S. typhimurium LT-2 ent-7 (an ent^- mutant) and phenol-chloroform-ether extracted material supported the growth of A. flavescens JG-9 (a hydroxamate auxotroph). Moreover, growth enhancement of V. vulnificus grown under iron stress was observed upon addition of concentrated culture extracts with both the ethyl acetate and phenol-chloroform-ether preparations. This study also demonstrated that V. vulnificus could not use enterobactin but Desferal (formation constant for iron of 10^{31}), the mesylate salt of deferriferrioxamine B (Whitten & Brough, 1971), would alleviate iron limitation. Andrus et al. (1983)

looked at the synthesis of siderophores by several pathogenic *Vibrio* species including *V. cholerae*, *V. fluvialis*, *V. parahaemolyticus*, *V. vulnificus*, *V. anguillarum*, and *V. alginolyticus*. Using the Arnow test (Arnow, 1937) to detect phenolates in low-iron culture supernatants, *V. cholerae*, *V. fluvialis*, *V. vulnificus*, and *V. anguillarum* all gave positive results. *V. vulnificus* also gave a positive $Fe(ClO_4)_3$, ferric perchlorate assay, (Atkin et al. 1968) and a positive Csåky test (Csåky, 1948). The ability to bind ferric iron at a low enough pH to destroy phenolate siderophores, as in the ferric perchlorate assay, suggests the presence of a hydroxamate siderophore. For every species tested, addition of iron suppressed the synthesis of the Arnow positive or perchlorate positive compound, a property characteristic of bacterial siderophore synthesis (Neilands, 1981).

As discussed in this thesis in PAPER 1, supernatant fluids from the wild type *V. anguillarum* 775 (which contain siderophore activity) were able to cross-feed strains carrying a mutation affecting siderophore activity [*V. anguillarum* 775::Tn*1*-5(pJHC-91) and *V. anguillarum* 775::Tn*1*-7(pJHC-W7)]. Working in collaboration with C. Andrus in Dr. S. Payne's laboratory at the University of Texas, Austin, I began to investigate the interaction of *V. vulnificus*, *V. anguillarum*, and *V. parahaemolyticus* with respect to siderophore activity (Andrus et al., 1983).

MATERIALS AND METHODS

Bacterial strains. Vibrio anguillarum 775 was isolated from Oncorhynchus kisutch in the state of Washington during an outbreak of vibriosis. V. anguillarum 775::Tn1-5(pJHC-91, iuc), 775::Tn1-6(pJHC9-8, iuc, iut) and H775-3(plasmidless) were described previously (Walter et al., 1983). V. anguillarum 2911 was from Salmo trutta in Scotland, V. anguillarum 1800 from Crassostrea gigas in Washington, V. anguillarum 514 from Japan, V. anguillarum 531A and 507 from the Atlantic coast, and V. anguillarum 286D from Washington. V. vulnificus 320 Lac^+, ade, gua, V. vulnificus 329 Lac^+, ade, gua, iuc, and V. parahaemolyticus M4-74-801 were gifts from Dr. S. Payne.

Bioassays for detection of siderophore activity. The ability of supernatant fluids or cultures to support the growth of iron uptake-deficient mutants in iron-depleted medium was tested by impregnating a sterile disk with 10 µl of supernatant fluid, or a streak of a culture from the growth of various organisms (to be tested for cross-feeding abilities). V. anguillarum and V. parahaemolyticus iron uptake proficient strains were grown in M9 minimal media containing 10µM EDDA for 48 hr at 22°C. V. vulnificus strains were grown in M9 minimal media containing 10 µM EDDA and 20 µg/ml adenine and 20 µg/ml guanine. The iuc mutants V. anguillarum 775::Tn1-5(pJHC-91) and V. vulnificus 329 were grown as above but with only 1 µM EDDA. Supernatant fluids to be tested were filter sterilized and made 10 µM in EDDA before application to the disk or culture streaking. A positive result was a zone of growth of the lawn strain around the disk or applied culture streak.

RESULTS

Vibrios exhibited diversity in their abilities to produce and utilize siderophores. Andrus et al. (1983) found *V. cholerae* supernatant fluids were able to stimulate growth for *V. fluvialis*, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* but not *V. anguillarum* 775 when these cultures were challenged by growth under iron limitation conditions. *V. fluvialis* supernatant fluids were able to stimulate growth for *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* but not *V. vulnificus* or *V. anguillarum* grown under iron stress. *V. vulnificus* supernatant fluids were able to stimulate iron stressed cultures of *V. fluvialis* but not *V. cholerae*, *V. anguillarum*, *V. parahaemolyticus*, or *V. alginolyticus*. *V. anguillarum* supernatant fluids were able to stimulate growth for iron stressed *V. fluvialis* but not *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, or *V. alginolyticus*. *V. parahaemolyticus* supernatant fluids stimulated growth of iron stressed *V. fluvialis*, and *V. alginolyticus* but not *V. cholerae*, *V. vulnificus* or *V. anguillarum*. Moreover, *V. alginolyticus* supernatant fluids stimulated growth in iron stressed *V. fluvialis* and *V. parahaemolyticus* but not *V. cholerae*, *V. vulnificus*, or *V. anguillarum*. Working with *V. vulnificus* 320 and *V. vulnificus* 329 (*iuc*), I found that strain 320 was able, as expected, to cross-feed strain 329 at 18°C and 30°C; however, *V. anguillarum* 775::Tn*1*-5(pJHC-91, *iuc*) and the plasmidless strain H775-3 were not able to be cross-fed by the culture supernatant or the culture strain of *V. vulnificus* 320. Moreover, *V. anguillarum* 775 supernatants or cultures

were not able to cross-feed V. vulnificus 329 (iuc) at either 18°C or 30°C. Both temperatures of 18°C and 30°C were used since the growth optima for V. anguillarum 775 of 18°C was not an optimum growth temperature for V. vulnificus. V. anguillarum 775 was able to grow (under iron stress) at 30°C but not as well as at 18°C. 37°C was not used because V. anguillarum 775 grows very poorly under iron stress conditions at this temperature possibly due to the spontaneous loss of the iron sequestration mediator, the pJM1 plasmid. I also found (data not shown) V. parahaemolyticus M4-74-801 cultures or culture supernatants were not able to cross-feed V. anguillarum 775::Tn1-5(pJHC-91, iuc) or H775-3. Andrus et al. (1983) found similar results by using cultures supernatants of V. vulnificus and V. parahaemolyticus and looking for a growth stimulation of V. anguillarum 775 cultures. Even though V. anguillarum was not able to be cross-fed by V. cholerae, V. fluvialis, V. vulnificus, V. parahaemolyticus, or V. alginolyticus, I have found a 'non-specific' cross-feeding of V. anguillarum 775 by other V. anguillarum isolates. V. anguillarum 286D, 2911, 1800, and 514 supernatant fluids or culture streaks were able to cross-feed V. anguillarum 775::Tn1-5(pJHC-91,iuc) as well as 775::Tn1-6(pJHC9-8, iuc, iut) and H775-3 (plasmidless). Figure 1 shows a representative positive result on culture streak 3 (V. anguillarum 514). These Vibrios 286D, 2911, 1800, and 514 are all virulent strains as tested in the salmonid fish system but do not possess a pJM1 class of plasmids. Two isolates, V. anguillarum 531A and 507, which do contain pJM1-like plasmids [very similar by restriction analysis (K. Kellerman, personal communication)] were found to cross-feed 'specifically' as would the wild type

V. anguillarum 775(pJM1); that is, they only cross-feed *V. anguillarum* 775::Tn*1*-5(pJHC-91) but not *V. anguillarum* 775::Tn*1*-6(pJHC9-8) or H775-3.

DISCUSSION

Virulent strains of *V. anguillarum* may possess the pJM1 plasmid or be plasmidless. Both types must be able to sequester iron from the hostile environment of the host where the free iron concentration is very low. In comparing *V. anguillarum* 775 wild-type cross feeding abilities with the pJM1-like plasmid containing strains *V. anguillarum* 531A and 507, the results were identical. All three of these strains cross-feed the *V. anguillarum* 775::Tn*1*-5(pJHC-91,*iuc*) strain but not the *V. anguillarum* 775::Tn*1*-6(pJHC, *iuc*, *iut*) or the plasmidless H775-3 strains (Fig. 1). However, cross-feeding of these three lawn strains [(Fig. 1), *V. anguillarum* 775::Tn*1*-5(pJHC-91, *iuc*), 775::Tn*1*-6(pJHC9-8, *iuc*, *iut*) and H775-3] occurred by the plasmidless virulent *Vibrios*, *V. anguillarum* 286D, 2911, 514, and 1800 (only 514 data shown). Toranzo et al. (1983) found that unlike the pathogenic *V. anguillarum* 775 (a Pacific Northwest isolate), strains pathogenic for striped bass from Chesapeake Bay did not contain any plasmids. However, their virulence also correlated with their ability to grow in the presence of nonimmune serum and under conditions of iron stress as does *V. anguillarum* 775 (pJM1). It has been known that *V. anguillarum* 775(pJM1) as well as H775-3 synthesize DHBA (dihydroxybenzoic acid, a precursor in enterobactin biosynthesis); however, this compound does not appear to interfere with the plasmid mediated system of iron uptake since under our conditions *V. anguillarum* H775-3 cannot grow under conditions of iron limitation (10 μM EDDA or 3 μM transferrin) whereas the plasmid containing strain does grow. I have

not determined what compounds are involved with the siderophore activity in the supernatant fluids of _V. anguillarum_ 1800, 514, 286D, and 2911; however, it certainly appeared to be different than the activity in the supernatants of _V. anguillarum_ 775(pJM1), 531A, and 507. Studies in Dr. J.H. Crosa's laboratory by K. Kellerman in collaboration with Dr. J. Neiland's laboratory at the University of California, Berkeley, have characterized the siderophore activity from _V. anguillarum_ 775(pJM1) as a heat stable activity that does not behave as a typical hydroxamate or phenolate. The biological activity may be extracted from iron-stressed culture supernatants of _V. anguillarum_ 775 by ethyl acetate, benzyl alcohol, and phenol-chloroform (K. Kellerman, personal communication). Recent results in our laboratory by Kellerman in collaboration with Dr. J. Sanders-Loehr at Portland State University, Portland, OR. have found the purified concentrated siderophore activity to undergo a spectral change when exposed to $Fe(ClO_4)_3$ suggesting the binding of iron by this purified extract (K. Kellerman, personal communication).

Figure 1. Bioassay

Panel A: Lawn strain containing *V. anguillarum* 775::Tn*1*-5(pJHC-91)

Panel B: Lawn strain containing *V. anguillarum* 775::Tn*1*-6(pJHC9-8)

Panel C: Lawn strain containing *V. anguillarum* H775-3.

Growth culture streaks applied to these lawns are;

1. *V. anguillarum* 775(pJM1)
2. *V. anguillarum* H775-3
3. *V. anguillarum* 514
4. *V. anguillarum* 775::Tn*1*-6(pJHC9-8)
5. *V. anguillarum* 531A (pJM1-like)
6. *V. anguillarum* 775::Tn*1*-5(pJHC-91)

A

Lawn strain containing pJHC91 plasmid

B

Lawn strain containing pJHC9-8 plasmid

C

Lawn strain containing plasmid-less strain

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APPENDIX B.

DNA Sequencing of Regions in the pJM1 Plasmid

INTRODUCTION

To further study the pJM1 iron uptake regions, I began DNA sequencing using the chain terminator method of Sanger et al. (1977). As reported in Paper 1 of this thesis, the pJM1 regions involved in iron sequestration are BamHI fragment 1 and possibly parts of fragments 5 and 6. Using restriction endonuclease analysis with molecular cloning techniques, parts of these regions were further analyzed. The Sanger method was my method of choice over the chemical sequencing method of Maxam & Gilbert (1980) since the chain terminator sequencing method is less laborious, less dangerous, and is reported to be more accurate since it depends upon the primed synthesis of a complementary radioactive copy of a single-stranded DNA template by the biological Klenow fragment of DNA polymerase I. Both methods are based on producing a population of radioactive polynucleotides in which each molecule has a common terminus (either 5' or 3'). From this population of radioactive molecules, a nested set of polynucleotides which extend from the common end to a base-specific termination are generated. Finally, the fragments are sized, usually by electrophoresis, in a high-resolution denaturing polyacrylamide gel. The sequence is then deduced from reading the autoradiograms.

The Maxam-Gilbert method is a chemical sequencing procedure. The DNA is attacked with reagents that first damage and then remove a base from its sugar. Since this exposed sugar is a weak point in the backbone of the DNA it can be cleaved completely from its 3' and 5' phosphates by

an alkali- or amine- catalyzed series of β-elimination reactions. The Sanger sequencing protocol uses dideoxytriphosphate incorporation into the growing oligonucleotide chain as the termination event. Because these dideoxytriphosphates contain no 3'-hydroxyl group, the chain cannot be extended further. Depending upon the mixture of, for example, ddATP to dATP, a mixture of fragments with different lengths all having the same 5' end terminating in ddA residues at the 3' ends are obtained.

To obtain a ready source of single-stranded template material the (F-specific rod-shaped filamentous) M13 phage (Pratt, 1969; Marvin & Hohn, 1969) host was exploited (Gronenborn & Messing, 1978). Since all viral genes in M13 are essential segments they could not be replaced by cloned DNA as was done with the lambda cloning vehicles (Williams & Blattner, 1980), so the M13 phage was altered in an intergenic region not involved in growth. Gronenborn & Messing (1978) originally constructed special M13 derivatives in which the *E. coli lac* operon gene containing the i gene (repressor), promoter, operator, and the first part of the Z gene, coding for the N-terminal 145 residues of β-galactosidase (the "α-fragment") were inserted into the intergenic space (507 bp of non-protein-coding DNA) between genes IV an II. The functional β-galactosidase enzyme contains N-terminal "α-fragment" and the C-terminal "w-fragment". If both of these fragments are present in the same cell they will associate sufficiently to give rise to β-galactosidase activity and a *lac*$^+$ phenotype. The "w-fragment" is produced by the host bacterial strain used, *E. coli* JM103 (Δ(*lac*-*pro*) *supE thi strA endA sbcB*15 *hsdR*4F *pro*$^+$ *lac*iz Δ M15 *tra*D36). The enzyme β-galactosidase will convert 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to a non-diffusible bright blue indole dye, so that in the

presence of X-gal and a gratuitous inducer, IPTG (isopropyl-thio-β-D-galactoside), *lac*$^{+}$ bacterial colonies are blue. Even though the M13 phage does not kill its host upon infection it does slow down the growth sufficiently to give rise to visible plaques on a bacterial lawn; therefore, infection of M13 into *E. coli* JM103 would give rise to blue plaques. Introduction of a piece of foreign DNA into a specifically constructed restriction site that interrupts the "α-fragment" gene of the phage will give colorless "white" plaques upon host infection. The utility of this phage in the sequencing method was greatly enhanced by the insertion of a short oligonucleotide (MCS-multipurpose cloning site) containing restriction endonuclease cleavage sites in the *lac* region of the M13 derivative. The MCS does not affect the *lac*$^{+}$ phenotype until a foreign piece of DNA is cloned into a cleavage site which then will interrupt the N-terminal coding region of the β-galactosidase gene and produce colorless plaques upon *E. coli* infection.

Because the intracellular replicative form (RF) of the M13 phage is a double-stranded supercoiled DNA the *lac* region, the MCS region, and the desired cloned fragment can all be inserted with recombinant techniques used with plasmid vehicles. The Sanger sequencing protocol calls for a single stranded DNA template with a primer that will allow continued strand elongation in an area of the cloned gene. A 'universal' primer has been employed that is complementary to the *lac* DNA region adjacent to the MCS. Thus polymerase elongation starts from the 'universal' primer and is terminated by the incorporation of a dideoxynucleotide into the growing polynucleotide chain. Each primed template is incubated in the presence of each of the four dideoxynucleotide:deoxynucleotide

triphosphate combinations. A nested set of fragments all starting at a distinct 5' end will be generated. Using DNA sequencing, I then began to study the pJM1 plasmid DNA sequences beginning my investigation around the iron sequestration region which includes pJM1 *Bam*HI fragment 1 and possibly fragments 5 and 6.

MATERIALS AND METHODS

Maintenance and growth of E coli. Escherichia coli JM103: Δlac pro, supE, thi, strA, sbcB15, endA, hspR4, F' traD36, proAB, $lacI^q$ Z ΔM15 was used to grow phage. E. coli JM103 was stored on minimal media plates M9 salts (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g Na Cl, 1 g NH_4Cl, 1ml of 1% vitamin B_1, and 10 ml of 0.01M $CaCl_2$ all in 1 liter total volume with dIH_2O(deionized water).

To support phage infection and growth, 2X YT (yeast tryptone) medium was used (16 g Bacto tryptone, 10 g Bacto yeast extract, 5 g NaCl in 1 liter of dIH_2O. Add 20 g of Difco agar per liter for plates and for the soft agar overlays 6 g of Difco agar per liter. All growth was at 37°C.

Maintenance and growth of phage. The Messing strains M13mp8 and M13mp9 (Messing & Víeira, 1982) were used as the phage vectors. For amplification of phage, one plaque was inoculated into 2 ml of a three hr culture of E. coli JM103 in 2X YT media and grown for 7 hrs with vigorous shaking at 37°C. The supernatant of this 2 ml preparation becomes the phage stock for that plaque and may be kept for years at -20°C. Another phage infection from the original plaque can be performed by infecting another 2ml -three hr culture of E. coli JM103 with 10μl of the supernatant, allow infection for 7 hrs then the supernatant can then be stored or sued to make single-stranded (ss) DNA for use in the Sanger sequencing procedure.

Preparation of template, ss-DNA. To 1.3 ml of the phage amplification supernatant add 200 μl of 27% PEG (polyethylene glycol) - 8000 in 3.3 M

NaCl in a 1.5-ml Eppendorf tube. Vortex and let sit for 60 min in the cold room. Centrifuge for 10 min in an Eppendorf microfuge. A small pellet should be visible. Suction off the supernatant and to the pellet add 0.65 ml of low-Tris buffer (10 mM Tris-HCl, pH7.5, 0.1mM EDTA). Resuspend while keeping on ice and then reprecipitate the DNA using 40 μl of 40% PEG-8000 and 80μl of 5M NaCl. Vortex and let sit at room temperature for 30 min. Again collect the precipitate by centrifugation in an Eppendorf microfuge and then decant by suction the supernatant. To the pellet add 200 μl of low-Tris buffer and then treat this DNA solution with equal volumes of TE (0.01 M Tris-HCl, 0.001 M Na_2EDTA, pH 8.1) equilibrated phenol, 1X, of TE saturated phenol in a 1:1 ratio with chloroform (24): isoamyl alcohol (1), 1X, of chloroform (24): isoamyl alcohol (1), 1X. Precipitate the DNA solution using 1/10th volume 3 M sodium acetate and 2.5 volumes of absolute ethanol at -70°C for at least 30 min. Collect the DNA precipitate by centrifuging for five min in an Eppendorf centrifuge. Decant. Wash the DNA pellet in 70% ethanol, spin again, decant and dry the pellet. Resuspend the dried DNA pellet in 20μl of dIH_2O and store at -20°C. This DNA is the ss-DNA template used in the Sanger 'dideoxy' sequencing procedure.

Preparation of replicative form DNA. Replicative form DNA is double-stranded (ds) covalently closed circular DNA. This ds-DNA is needed as a substrate for restriction enzyme analysis of inserted DNA in the M13 phage vectors. Using the phage amplification supernatant (see Materials and Methods: Maintenance and growth of phage) add 100μl to 40 ml of a 3 hrs *E. coli* JM103 culture in 2X YT media. Let the infection progress at 37° C for 5 hrs. Infected cells which contain the ds-DNA are collected by centrifugation on a Beckman J2-21, 5000 x g for 5 min at

4°C. The supernatant fraction can be saved at -20C° to later prepare ss-DNA for the template. The cell pellet is resuspended in sucrose buffer (25% sucrose in 0.05 M Tris-HCl, pH 8.0, 0.01 M EDTA) to a volume of 1.0 ml. Keep at 4°C. Add 0.3 ml of 5 mg/ml lysozyme in 0.05 M Tris-HCl, pH 8.0, 0.01 M EDTA made fresh. Gently mix and wait 5 min at 4°C. Then add 0.6 ml of 0.25 M Tris-HCl, pH 8.0, 0.25 M EDTA and subsequently 0.025 ml of 10 mg/ml RNAse solution (10 mg/ml in 0.01 M sodium acetate pH 5.0; boil for 2 min then store at -20°C in small aliquots). Incubate 5 min then add 2.5 ml of Tris-EDTA-Triton (0.05 M Tris-HCl, pH 8.0, 0.01 M EDTA, 2% Triton X-100) for a further 10 min. Mix gently. Centrifuge at 17,000 x g for 20 min at 4°C. The pellet was resuspended in dIH_2O to a 5 ml total containing 0.3 ml of a 10 mg/ml ethidium bromide solution and enough cesium chloride for a refractive index of 1.3916. The sample was then centrifuged in a vertical rotor (VTi 65) for 24 hrs at 15°C at 55,000 rpm. The lowest band will be RF-ds-DNA, and the top band chromosomal DNA, there may be a band of intermediate density which is the ss-DNA. Collect the ds-DNA. Extract the ethidium bromide with 5 extractions of isopropanol saturated with CsCl in TE. Dialyse the DNA against 2-500 ml volumes (vol) of 6 mM Tris pH 7.5 with the total dialysis time of 2 hrs at 4°C with a buffer change at 1 hr. Ethanol precipitate the DNA using 1/10th vol 3 M sodium acetate and 2.5 vol of absolute ethanol for 30 minutes at -70°C. Collect the DNA precipitate by a 5 minute Eppendorf microfuge spin. Wash the pellet in 70% ethanol, dry the pellet and resuspend the DNA in 200 µl of 20 mM Tris pH 8.0. Freeze at -20°C.

Phage Plaques.

1. Plaque purification. Pour a plate that contains 20 ml of 2X YT (2% agar) in a petri dish and let harden. Pour an overlay that contains 3 ml of 2X YT (0.6% agar), 50 µl of X-gal (5-dibromo-4-chloro-3-indolylgalactoside). This is a 2% solution in dimethylformamide that should be protected from light and stored at 4°C, 20 µl of IPTG (isopropylthiogalactoside, 200 mM in dIH_2O), and 0.3 ml of a 3 hr 2X YT culture of JM103. Pour this overlay and let harden. Apply 1 µl of a phage amplification supernatant from a 2 ml culture and then spread across the plate using a sterile strip of 3MM Whatman filter paper. Once one streak is finished apply a second strip to the end of the original streak to obtain an inoculum then start a second streak. Doing this, one is diluting the sample to obtain isolated plagues. About 6 strips are used per sample. Incubate plates at 37°C overnight.

2. Phage plaques after transformation. To a plate that contains 20 ml of bottom agar (2X YT) pour an overlay that contains 3 ml of 2X YT (0.6% agar), 50 µl of X-gal, 20 µl of IPTG, 0.3 ml of a 3 hr 2X YT *E. coli* JM103 culture, and 0.2ml of the transformation preparation. Incubate plates at 37°C overnight. Plaque with inserts should be colorless.

3 hr 2X YT E. coli JM103 culture. To 20 ml of 2X YT medium add one colony of *E. coli* JM103 (from a minimal M9 medium agar plate that had been grown at 37°C for 48 hrs) and grow at 37°C for 3 hrs or until the cell optical density was about OD_{600}= 0.3. These cells may be used immediately or kept on ice for several hours before use. The minimal agar plates that the *E. coli* JM103 was taken from were transferred monthly and kept at 4°C. Frozen stocks of *E. coli* JM103 that had been grown in minimal media were made by adding glycerol to a 20% final

concentration and freezing at -70°C.

Preparation for cloning desired DNA inserts into the M13 phage vectors.

1. Preparation of inserted and vector DNA. *Eco* RI fragment 7 of pJM1 plasmid DNA was shotgun cloned into the vector pKY2700 (Ozaki et al., 1982) into the *Eco*RI site. Purified DNA was made from this clone, using the Birnboim and Doly (1979) method modified as described in the Cold Spring Harbor Laboratory Manual (Maniatis et al., 1982) and various subfragments of the pJM1 DNA were ligated into the M13mp8 and M13mp9 vectors. The ligation experiments were carried out at 15°C for 24 h using 0.05 U of T4 DNA ligase (Bethesda Research Laboratories) (Walter et al., 1983).

2. Preparation of competent cells. The method of Shigesada (1983) was used. The efficiency of tranformation was about 10^6 to 10^8 transformants/ μg of DNA. ϕ medium (20 g Bacto tryptone, 5 g of yeast extract, 5 g of $MgSO_4$ and dIH_2O to 1 liter, pH 7.6) was filtered through a 3MM Whatman filter and autoclaved. Solution I: 30 mM K-acetate 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8 adjusted with 0.2 M acetic acid filter sterilize. Solution II; 10 mM MOPS (morpholinopropane sulfonic acid), 75 mM $CaCl_2$, 10 mM RbCl and 15% glycerol, pH 6.5 with dilute KOH. An overnight *E. coli* K23 cultured was used to inoculate 100 ml of fresh ϕ medium (1% inoculum). Inculate cells at 37°C until OD_{540} 0.5. Cells were left on ice for 5 min and then harvested by centrifugation at 4000 rpm x g for 5 min at 4°C. The cell pellet was resuspended in 20 ml of Solution I and put at 4°C for 5 min., harvested as before and resuspended the pellet in 4 ml of Solution II. The preparation was left standing on ice for 15 min and then the cells

were dispensed in 0.2 ml aliquots into prechilled test tubes. Freeze using ethanol-dry ice and keep at -70°C.

3. Transformation procedure. The frozen competent cells were thawed at room temperature and then put on ice for 15 min. The DNA was added (<100 ng) and the preparation left on ice for another 20 min. Heat shocked at 42°C for 90 sec and then returned to ice for 2 min. The DNA/cell mixture is then ready to be added to the top agar in the phage plaqueing after transformation procedure (see Phage Plaqueing #2).

Phage dot hybridization. To screen a large number of recombinants, 10 μl of the phage supernatant to be tested are spotted directly on a sheet of nitrocellulose paper (Hu & Messing, 1982) Air dry the filter for 2 h at room temperature then bake in a vacuum oven at 80°C for 2 h. This filter is ready for hybridization to a radioactive probe.

Hybridization. Phage dot nitrocellulose filters were next placed into plastic bags containing 5X SSC (1X SSC= 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, Denhardt solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin [Denhardt, 1966]), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After 3 h at 37°C, the appropriate heat-denatured, ^{32}P-labeled plasmid DNA (10^6 cpm) was added and hybridization was carried out by incubation in the same preincubation solution with carrier salmon testes DNA at 100 μg/ml for 16 hr at 37°C. The blot was rinsed quickly in 200 ml of 5X SSC, 0.1% SDS (sodium dodecyl sulphate) solution twice. It was then washed for two one hour periods at 65°C in 500 ml of the above solution each wash. The blot was then quickly rinsed twice with 200 ml of 2X SSC solution at room temperature. The blot was then air dried and was ready for autoradiography.

Autoradiography The blot was placed between two intensifying screens with Kodak XRP-1 film. The film was exposed to -70°C for a varying amount of time.

Nick-translation. ^{32}P-DNA probes were prepared by the nick-translation method of Rigby et al. (1977). Add at 4°C, 5 μl of dATP (NEN specific activity 800 Ci/mmol), 5 μl of 10X nick translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1 mM DTT, 500 μg/ml BSA), 1 μl of 1 mM unlabeled d NTPs (nM of each d NTP (N = G,A,T, or C); 0.5 μg of DNA; dIH_2O to make 49 μl. Then add 0.5 μl of DNAse I (1/1000 dilution of a 1 mg/ml solution with 1X nick translation buffer in 50% glycerol), 0.5 μl of DNA polymerase I (approx. 5 units), mix well and incubate for 1 h at 15°C. Stop the reaction by adding 10 μl of 0.5 M EDTA pH 8.0. Incubate the reaction mix at 60°C for 10 min and then load on a Sephadex G75 column (use 1 ml plastic syringe). Elute the DNA with TE buffer collecting 30-50 μl fractions. The ^{32}P-DNA peaks were collected in fractions 8,9 and 10 while the nucleotide peak was in fractions 15 to 20. Values of 10^6 cpm/fraction were used for filter blot hybridizations.

Large Scale Birnboim and Doly Method (Maniatis et al., 1982) 500 ml culture of *E. coli*, containing the desired plasmid, was grown with the appropriate antibiotic at 37°C overnight. The cells were harvested by centrifugation at 5,000 x g for 20 min. The pellet was resuspended in 10 ml of Solution I containing 5 mg/ml lysozyme (Solution I: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA). Incubate at room temperature for 5 min after vortexing. Add 20 ml of freshly made Solution II: 0.2 N NaOH, 1% SDS, mix gently and let stand on ice for 10 min. Add 15 ml of ice-cold 5 M potassium acetate (pH 4.8) and let stand on ice for 10 min. Centrifuge on a Beckman J2-21 at 48,000 x g for 20 min at 4°C. To the

supernatant add 0.6 volumes of cold isopropanol. Mix well and let stand at room temperature for 15 min. Recover the DNA by centrifugation at 12,000 x g for 30 min at room temperature. Discard the supernatant and wash the pellet in 70% ethanol. Dry the nucleic acid pellet in a vacuum desiccator. Dissolve the pellets in a 3 ml of TE buffer. Add 0.3 ml of 10 mg/ml ethidium bromide and enought TE and cesium chloride to make a total volume of 5 ml and a refractive index of 1.3916. Centrifuge in a vertical rotor (VTi65) for 16 hrs at 15°C at 55,000 rpm. Collect the bottom plasmid band. Extract the ethidium bromide with 5 extractions of isopropanol saturated with CsCl in TE. Dialize the DNA against 2-500 ml volumes of 6 mM Tris pH 7.5 with the total dialysis time of 2 hrs at 4°C with the buffer change at 1 hr. Ethanol precipitate the DNA using 1/10 vol 3 M sodium acetate and 2.5 vol of absolute ethanol for 30 minutes at -70°C. Collect the DNA precipitate by a 5 min Eppendorf micorfuge spin. Wash the pellet in 70% ethanol, dry the pellet, and resuspend the DNA in 200 µl of 10 mM Tris pH 8.0. Freeze at -20°C.

Elution of DNA from an Agarose Gel. Electrophoresis of cleaved DNA was performed in a horizontal 0.5% agarose gel in Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid, pH 8.2). The restriction fragment bands desired were cut out of the gel and placed into large dialysis bags. Electroelution of the DNA from the agarose into the dialysis bag was carried out using a Tris borate buffer (1/10 the concentration specified above) for 3 hr at 200 V (20mamp). After reversing the current for 10 sec, the dialysis bag contents were placed into Nalgene centrifuge tubes, and agarose was sedimented by centrifugation at 17,000 x g, at 4°C for 10 min. The DNA-containing supernatant was made 0.3 M in sodium acetate, and the DNA was

precipitated by adding 2.5 vol of ethanol at -20°C. The DNA was then sedimented by centrifugation for 1 hr at 29,000 x g at -10°C, washed with 70% ethanol (-20°C) and the dried pellet resuspended in a desired volume of 6 mM Tris-hydrochloride, pH 7.5.

Sequencing. DNA sequencing was performed using the Sanger 'dideoxy' method, (Sanger et al., 1977; Sanger & Coulson, 1978) as directed by the NEN (New England Nuclear) DNA Sequencing System, kit NEK-023. Hybridizing the primer to the template add 2 µl to ss-template DNA, 1 µl (0.2 picomoles) of a 15 mer primer (2.6 ng/µl) 3'OH-ATGTTGCAGCACTGA-OH5', 1.5 µl of H-buffer (100 mM Tris-HCl, pH 7.9, 600mM NaCl, 66 mM $MgCl_2$), and 8.5 µl of dIH_2O to a 0.5 ml microfuge tube at room temperature and incubated at 55°C for 5 min to anneal the primer to the template. Equilibrate the tube slowly to room temperature. To each hybridization mixture add 2 µl of dATP [α-^{32}P] at 10 µCi/µl, 1 µl of freshly prepared 0.2 M DTT (dithiothreitol) and 1 µl of the Klenow fragment of DNA polymerase 1. All additions are carried out on ice. Four 0.5 ml micorfuge tubes, one each for the G, A, T, and C reactions are set up for each hybridization reaction. To each tube add 3 µl of the hybridization reaction mixture and the appropriate mixture of the 'deoxy/dideoxy' mixture eg. dG/ddG, dA/ddA, dT/ddT, dC/ddC so that the final nucleotide concentration in µM is:

Nucleotide Final Concentration (μM) (before chase addition)

Nucleotide	G	A	T	C
dCTP	36	25	36	3.6
dGTP	3.6	25	36	36
dTTP	36	25	3.6	36
dATP, [α-^{32}P]	1.5	1.5	1.5	1.5
ddATP	-	50	-	-
ddCTP	-	-	-	200
ddGTP	200	-	-	-
ddTTP	-	-	400	-

Deoxynucleoside triphosphates

dG = deoxyguanosine

dA = deoxyadenosine

dT = deoxythymidine

dC = deoxycytidine

Dideoxynucleoside triphosphates

ddG = dideoxyguanosine

ddA = dideoxyadenosine

ddT = dideoxythymidine

ddC = dideoxycytidine

This reaction is incubated at 37°C for 30 min. Add 1 μl of chase (40 μl each of 10 mM dGTP, 10 mM dATP, 10 mM dTTP, 10 mM dCTP, and dIH_2O) and mix. Incubate at 37°C for 30 min then add 10 μl of stop mix (0.05% each of xylene cyanole FF and bromophenol blue 99% deionized formamide, 10 mM EDTA, 10 mM NaOH). Mix. Incubate tubes at 95°C for 3 min then put on wet ice immediately. Load 3 μl onto an 8% polyacrylamide gel (35 x 43 x 0.04 cm) containing 7M urea for sequence analysis.

The acrylamide gel preparation:

1. The acrylamide stock is 38% BioRad acrylamide and 2% BioRad bisacrylamide in water.
2. 10X TBE (121 g Tris OH, 54 g Boric Acid, 7.45 g sodium EDTA, water to one liter)
3. The gel: 40 gr Isolab urea, 24.8 ml dIH_2O, 8 ml of 10 x TBE (see #2), 16 ml of acrylamide stock (see #1), 0.064 g of BioRad dry ammonium persulphate and 20 µl of TEMED (BioRad).

The gel was pre-electrophoresed for ½ hr to 1 hr at 1700 V. After loading the samples the electrophoresis was carried out at 40-50 mA. (2000 V) and was terminated when the bromophenol blue dye reached the bottom of the gel for the short run (approx 2 hrs) and for the long run after a 3½ to 4½ hr run time. Kodak XAR-5 (35 x 43 cm) film was added to the gel and exposed in a film holder at -70°C for less than 12 hrs. The autoradiogram of the sequencing gel showed four ladders of bands, each ladder for a nucleotide being sequenced G,A,T or C. Bands near the bottom of the gel are images representing DNA synthesis closest to the 3' end of the primer, and bands at the top of the gel represent DNA synthesis furthest from the primer.

RESULTS

1. Repeated sequences

I originally noticed that a nick-translated agarose purified pJM1 BamHI fragment 1 gave a positive hybridization reaction with pJM1 BamHI fragments 1, 5, and 7. This was at first confusing and I thought that the agarose purified fragment might not be pure, thus I cloned fragment 1 into the vector pBR325 (Bolivar, 1978) and used this plasmid as the probe. The results were again the same. At this time Mr. Yasutami Mitoma was visiting our laboratory from Myasaki University in Japan and he obtained similar results using a probe containing pJM1 EcoRI fragment 1 in the vector pBR322 (Bolivar et al., 1977). This probe hybridized with the pJM1 EcoRI fragments 1,5,7,8,11. Thus, it was suggested that there were sequences in these other fragments that were recognized in the probe fragment and so our laboratory began to study these 'repeated sequences'. Mr. Jose' Perez-Casals and Mrs. Patricia Perez-Casals in our laboratory have recently located on the pJM1 plasmid one of the repeated sequences, Type 7, on the pJM1 EcoRI fragment 7 within the XhoI - ClaI segment of 1.5 kb. Another 'Type 7' sequence was found on EcoRI fragment 8 which lies within pJM1 BamHI fragment 1. These two 'Type 7' repeated sequences (Y. Mitoma, J. Perez-Casal, M.A. Walter, and J.H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B175, p. 46) are at the borders (Fig. 1, panel 3) of the iron sequestration region that was earlier determined by Tn1 transposition analysis (Walter et al., 1983). This finding has tremendous implications in light of other

findings that many transposable elements are bordered by homologous sequences (Kleckner, 1977, Calos & Miller, 1980). Thus I have started to analyze the 'Type 7' sequence on pJM1 EcoRI fragment 7 that was found between XhoI and ClaI. The sequencing of this 'Type 7' sequence was approached using two different clones. The large HindIII to EcoRI fragment of the pJM1 EcoRI fragment 7 of about 1.8 kb was cloned into the M13mp8 (clone:1-8-2; Fig. 1, panel 1 region A; Fig. 2) and M13mp9 (clone:1-9-2) vectors. Moreover the XhoI to PvuII fragment of about 0.2 kb (Fig. 1, panel 1 region C) was force cloned into M13mp9 [clone: 9-RS-XPv-(775)6]; that is, the insert XhoI sticky end was ligated to the vector SalI sticky end to generate a TaqI site while the blunt ended PvuII insert end was blunt end ligated into the SmaI end of the vector. These two different inserts overlap in the HindIII to PvuII site. One M13mp8 clone containing the 1.8 kb HindIII to EcoRI clone, 1-8-2, has been sequenced starting at HindIII for over 230 bases (Fig. 2). M13mp9 clone [9-RS-XPv-(775)6] containing the 0.2 kb XhoI to PvuII fragment has been sequenced starting from the PvuII/SmaI hybrid site and proceeding to the XhoI site (P. Perez-Casals, personal communication). The sequencing of these two clones revealed that the strand being sequenced on one was the complementary strand to the other, thus I have confirmed the sequence between the HindIII/PvuI fragment of pJM1 EcoRI fragment 7 that has been determined by hybridization analysis to contain the 'Type 7' repeated sequence.

The sequence reads starting from the *Hind*III site going toward the *Pvu*II site of clone 1-8-2 (Fig. 2) 5'to 3' and on 9-RS-XPv(775)6 from the 3' to 5':

```
  HindIII
5'AAGCTT'GTTCAGCGCTTTGATCATAGCGTAAGTCT
3 TTCGAA CAAGTCGCGAAACTAGTATCGCATTCAGA

5'CGCCAACCTGAGCATTGTAATTTCTTAGGCTTAATTT
3'GCGGTTGGACTCGTAACATTAAAGAATCCGAATTAAA

            PvuII
5'CCCACCTAG'CAGCTG'TTTTACTCGGTACATTGCT
3'GGGTGGATC GTC 5'

5'GTCTCTGATAGTGAGCGCTTATGATAGCCATACCGC

5'TTTTTCATTTCTTGTTGGAGCCGTAGAGCTTTTGACA

5'CTACTCGTAATTACGAGGGTGCCCTTGTTCCAGATGA

            5'GCCTTTTGGTAGGA 3'
```

It is interesting to note (Fig. 1, panel 3) that this region XhoI to PvuII in pJM1 BamHI fragment 5 is 200 base pairs away (at the PvuII end) from the Tn1 insertion in V. anguillarum 775::Tn1-11(pJHC-W11), an iron uptake proficient derivative.

Another HindIII to EcoRI fragment, the smaller fragment, of pJM1 EcoRI fragment 7 (Fig.1, panel 1 region B) was cloned into M13mp8 and M13mp9 vectors. This area of the pMJ1 plasmid is contained within BamHI fragment 1 but just outside the pJM1 HindIII fragment 14 that is where the Tn1 insertion took place to make the iuc mutant V. anguillarum 775::Tn1-5(pJHC-91). Clone 1-8-3 in M13mp8 and clone 1-9-3 in M13mp9 contain the HindIII to EcoRI inserts of approx. 290 bases. Clone 1-8-3 (reading from the HindIII site to the EcoRl site) and clone 1-9-3 (reading from the EcoRI site to the HindIII site) sequences read:

```
  HindIII
5''AAGCTT'TGTGCAAATGACAATGGTAGCTT

5'ACTACCTATAGAGGTTGAACCGTGTGGGAGA
                               3'CT

5' GCCATATCTACTGGGCTTATTGCGTGACGA
3' CGGTATAGATGACCCGAATAACGCACTGCT

5'CTTATCGGTTAGCGATACGCAGCGAGCGAG
3'GAATAGCCAATCGCTATGCGTCGCTCGCTC

5' TGCTTGTGCGCTTGGTGATTTGAAAAAGT
3' ACGAACACGCGAACCACTAAACTTTTTCA

5'CATTAAAGCTGATTTCAATATCATGGTTATT
3'GTAATTTCGACTAAAGTTATAGTACCAATAA

5'CATTAAGTTACCAATGATTCTTGTTGCTATA
3'GTAATTCAATGGTTACTAAGAACAACGATTA

3'TTGCTCACTGGTGGGTAAAGTTTTTTTAGTA

3'GTGGATTGAGTTGGAGCCCCCACGTAGTTC

                     Eco RI
   3'TGACCCGAGA'CTTAAG'
```

2. The Tn1 insertion in the siderophore activity minus, receptor activity positive V. anguillarum 775::Tn1-5(pJHC-91) is located in BamHI fragment 1 which contains HindIII fragment 14. HindIII fragment 14 of pJM1 restricted DNA becomes pJHC-91 HindIII fragment 1 because the Tn1, which is not cleaved by HindIII, increases the size of HindIII fragment 14 by 4.9 kb and the fragment migrates slower in the agarose gel. This fragment of 6.9 kb was eluted from a 0.5% agarose gel and cut with PstI, a restriction enzyme that does not cut the HindIII pJM1 fragment 14 but does have three sites in the Tn1 sequence. Since Tn1 is very similar to Tn3 (Heffron et al., 1979; Fred Heffron, personnal communication) with respect to its DNA sequence one would expect Tn1 to have a PstI site at base 4494 (Tn3) in the bla gene, at base 1645 (Tn3) in the tnpA gene and at base 987 in the trpA gene (note: Tn3 has 4957 bp). Thus I ligated the M13mp8 and M13mp9 doubly restricted with HindIII and PstI to the pJHC-91 HindIII fragment 1 cut with PstI. Those clones that, by the phage dot hybridization experiment, hybridized with the ^{32}P nick-translated plasmid, pJM1 EcoRI fragment 7 in pKY2700 (Fig. 1, panel 2), were further studied by DNA sequencing. It should be emphasized that positive clones will be those that contain pJM1 DNA and/or those that contain sequences that will hybridize with the Tn3 sequences in pKY2700. Several clones gave a positive hybridization result. One clone, A-23 (Fig. 1, panel 1 region D 2), hybridized very strongly with the probe and thus I began a sequencing analysis starting from the PstI site. The preliminary sequencing results show that the sequence for at least 150 bp in clone A-23, that contained part of Tn1, is the same as the Tn3 sequence in the bla gene at base 4494. However, because I am only able to sequence in from a cloning site about 200+ bp, I need to perform subcloning of this

insert to obtain overlapping sequences. To enable this procedure I have purified A-23 RF DNA so that it may be used in further cloning experiments. The clone A-23 sequence starting from the PstI site reads:

PstI
5'CTGCAG'CAATGGCAACAACGTTGCGCAAACTATTA

5'ACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAA

5'TTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGA

5'CCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTA

5'TTGCT 3'

The 'ori' (origin of replication) region of the pMJ1 plasmid is on BamHI fragment 2 (Yasutami Mitoma, personnal communication). Moreover, a smaller segment of 2 kb that contains an internal HindIII site will support replication in a polymerase polA mutant (Kingsbury,1973). Cleavage at the HindIII site on this 2 kb fragment no longer allows replication of a pBR322 vector containing either HindIII cut segment in a polA host. For replication, ColE1 replicons such as pBR322, require the host-encoded enzyme DNA polymerase I (product of the polA gene) (Kingsbury, 1973). Thus, selection for an antibiotic marker carried on pBR322 in a polA host after transformation would suggest the presence of an 'ori' region capable of supporting the replication of the chimeric plasmid in the polA host.

I have cloned into M13mp8 a _Hind_III/_Sal_I cut of the pJM1 _Bam_HI fragment 2 region. The following sequence was obtained for clone YR-8-SH-3:

```
    HindIII
5''AAGCTT'CCAGTGTTGATGGTGGCTTGGCTTTTTTT

5'GTTTTTCAAGTTGTTGCGCGGCAATGCAACTACTTGG

5'TAAATGATCGCTTCTATTACTTTAGAGGTGGGTCAAT

5'GATATTTTTCAAGAAATTGGGTAAGCACTTTGGCGAG

5'TGCAAGAATCTTTCGTTGCTGTTACGCACAGTTTTAA

5'TACCAGGGAGGTATCCGTTATGTAGATCCTTTTCCAG

          5'TACCTGGCCGATTC 3'
```

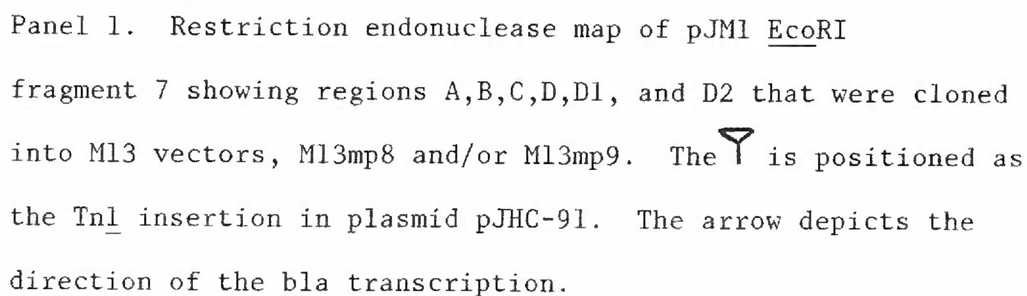
DISCUSSION

DNA sequencing is an advanced tool of molecular biology that allows a further characterization of a system. Through a combination of physiologic and genetic studies, many consensus sequences have been found in DNA and RNA molecules. Many of these appear to be necessary, for example, for proper enzyme function, template transcription, and translation. When one obtains sequence information in a molecule such as DNA, searches are usually made for sequences of known function. Mekalanos et al. (1983) found through DNA sequencing that a sequence of nucleotides, TTTTGAT, was found tandemly repeated 3 to 8 times preceding the cholera toxin _ctx_A gene. Physiological studies have shown that the _V. cholerae_ strain 569B, which carries eight TTTTGAT sequences upstream from its toxin operon, produced two orders of magnitude more toxin than strains that do not carry as many repeats; moreover, cloning experiments in both _E. coli_ and _V. cholerae_ suggest that these 8 bp repeated sequences are part of a toxin regulation system unique to _Vibrio_. Thus at the DNA sequencing level, one has the opportunity to detect small differences and small similarities when comparing structural genes with similar function; such as the _E. coli elt_ gene (LT toxin) and the _V. cholerae ctx_ gene (cholera toxin). In reviewing the sequences that I have generated, I did not find any exact sequences of TTTTGAT; however, in the sequence of clone 1-8-2 I have found these sequences: _C_TTTGAT, TTTT_C_AT, TTTTGA_C_, and TTTTG_G_T which all differ by only one base (underlined); this is a ratio of 4 sequences per 460 total bases or 230 bases per strand. For comparison, I was able to locate 11 sequences,

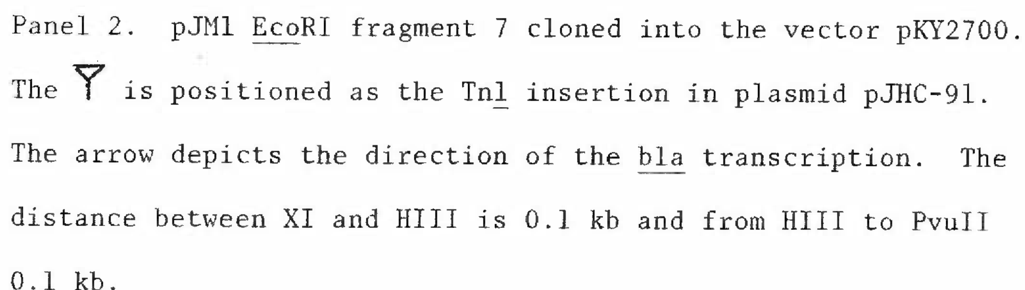
either a perfect match or one base substitution, in the 9914 bases (4957 bases per strand) of Tn3. Moreover, in this sequence of clone 1-8-2 there was also a perfect Pribnow (Pribnow, 1975a; 1975b) consensus sequence (TATNATN) of TATGATA after the PvuII site going toward the 3'end. Upon examination of clone 1-8-3(1-9-3) I also found a perfect consensus Pribnow sequence of TATCATG and one of the TTTTGAT-type sequences 5'TTTTGAA3' (mismatch underlined) in the 576 bases or 288 base pair sequence. The clone YR-8-SH-3 contains 5 TTTTGAT type sequences TGTTGAT, TTTTGTT, 3'TTTTGTT5', 3'TTTTTAT5' (these sequences are in the opposite orientation of the 5'TTTTGAT3', Mekalanos et al. 1983), and TTTTAAT. The Pribnow consensus sequence of TATCATT was also found (complementary strand to the one shown).

Even though this approach is informative one must proceed cautiously since it remains difficult to predict from DNA sequences which regions of the DNA will actually be utilized as a promotor or regulatory sequence. However, once a DNA sequence has been determined; such as, the area of the repeated sequence 'Type 7', the ori region of the plasmid, or regions in the iron sequestration area, one can then compare with other sequences of similar function.

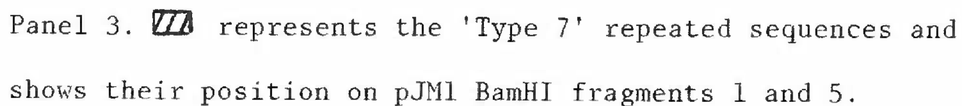
Figure 1. Detailed restriction map of pJM1 regions involved in the DNA sequencing analysis.



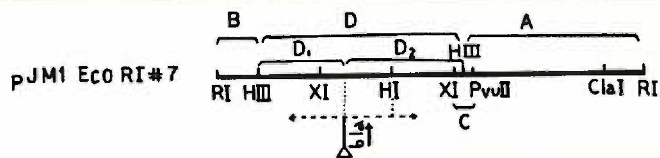
Panel 1. Restriction endonuclease map of pJM1 EcoRI fragment 7 showing regions A,B,C,D,D1, and D2 that were cloned into M13 vectors, M13mp8 and/or M13mp9. The Y is positioned as the Tn1 insertion in plasmid pJHC-91. The arrow depicts the direction of the bla transcription.

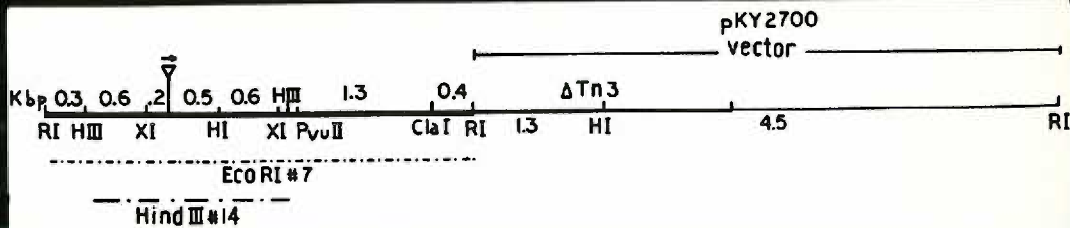


Panel 2. pJM1 EcoRI fragment 7 cloned into the vector pKY2700. The Y is positioned as the Tn1 insertion in plasmid pJHC-91. The arrow depicts the direction of the bla transcription. The distance between XI and HIII is 0.1 kb and from HIII to PvuII 0.1 kb.



Panel 3. ▨ represents the 'Type 7' repeated sequences and shows their position on pJM1 BamHI fragments 1 and 5.


PANEL 1
B
D
A
D₁
D₂
HIII
pJM1 EcoRI#7
RI HIII XI HI XI PvuII ClaI RI
C


PANEL 2
pKY2700
vector
Kbp 0.3 0.6 .2 0.5 0.6 HIII 1.3 0.4 ΔTn3
RI HIII XI HI XI PvuII ClaI RI 1.3 HI 4.5 RI
EcoRI #7
HindIII #14

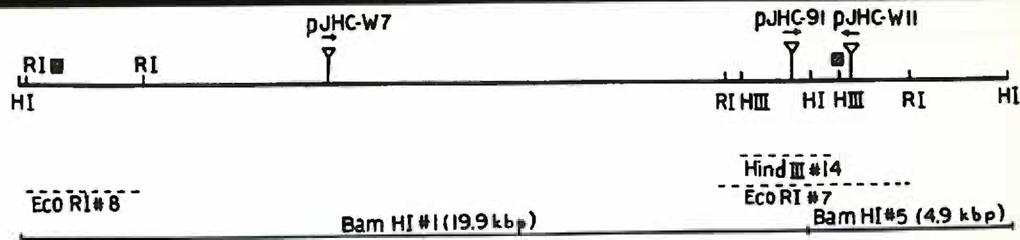

PANEL 3
pJHC-W7
pJHC-91 pJHC-W11
RI RI
HI
RI HIII HI HIII RI HI
HindIII #14
EcoRI #7
EcoRI #8
Bam HI #1 (19.9 kbp)
Bam HI #5 (4.9 kbp)

Figure 2. DNA sequencing autoradiographs of clone 1-8-2.

Autoradiograph 1. A 2 hour electrophoresis run. Lanes G,A,T,C, represent guanine, adenine, thymidine, and cytosine bases in the DNA.

Autoradiograph 2. A 3½ hour electrophoresis run. Lanes G,A,T,C, represent guanine, adenine, thymidine, and cytosine bases in the DNA.

1 2

G A T C G A T C

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V. SUMMARY AND DISCUSSION

The great philosopher Aristotle wrote, "All men by nature desire to know." The collection of experiments in this thesis represent one path used to explore a system of bacterial adaptation, a mechanism of virulence. Adaptation is the process of modification of a living organism to adjust to the conditions of its environment. My thesis project has been involved with the adaptive mechanism of iron sequestration in the marine fish pathogen Vibrio anguillarum 775. Iron (Fe), ranking fourth in abundance in the earth's crust, comprises about 5% of the weight of igneous rocks. It ranks number 9 in abundance among the elements in terms of cosmic abundance and number 23 in abundance in seawater. This transition metal, with its $3d^6 4s^2$ electron configuration, forms (Fe^{2+}) and (Fe^{3+}) ions. It is the acquisition of this metal that enables certain bacteria to cause a disease state. What determines a disease state? Disease can be equated to the product (virulence and the number of organisms) divided by the host defenses. Virulence may be defined as the degree of pathogenicity, that is, the infectivity (ability to colonize a host) and the severity of the disease produced. And, virulence factors are those components of an organism whose loss specifically impairs virulence but not viability. In general, bacterial virulence factors promote growth in the antagonistic environment of the host defense mechanisms. An iron acquisition system thus can be defined as a virulence factor, an adaptive mechanism used to satisfy a

nutritional requirement as well as allowing those organisms that possess such systems a competitive edge.

The iron uptake system I have been studying is mediated by the 65 kb virulence plasmid, pJM1. This low copy number plasmid (Crosa & Hodges, 1981) contains an origin of replication on BamHI fragment 2 (Yasutami Mitoma, personal communication) that can enable a ColE1 replicon to replicate in an *E. coli polA* strain. This suggests, that unlike ColE1 replicons, the *V. anguillarum* pJM1 origin of replication does not need the DNA polymerase I enzyme. Further experiments to complete the DNA sequence of the 'ori' region (2 kb) will allow comparison with other 'ori' regions that have been sequenced (Stalker et al., 1979; Tomizawa et al., 1977). This large plasmid does not appear to be conjugative (J.H. Crosa, personal communication) nor code for any antibiotic resistance characteristics (Y. Mitoma, personal communication); however, Crosa (1980) found that the possession of this plasmid enables *V. anguillarum* 775 to grow under conditions of iron limitation.

I have investigated this plasmid mediated iron uptake system using a physiologic bioassay to characterize two types of activities in the wild type parental strain: a siderophore activity and a receptor activity for the ferrisiderophore complex. This bioassay employed the use of a differential media containing the iron chelator, EDDA, which would only allow *V. anguillarum* to grow when a complete iron sequestration system was present. Derivatives of *V. anguillarum* 775 were generated by isolating ampicillin resitant *V. anguillarum* exconjugants that contained the TnA transposon, Tn*1*, inserted into the pJM1 plasmid. This procedure (Walter et al., 1983) as described in the Paper 1 section of this thesis, gave several Tn*1* insertions on the pJM1 plasmid. Some of these

insertions are quite stable but other derivatives 'throw off' deletion plasmids. I only characterized one deletion plasmid, however, there have been several produced and this could be an area of active research for the laboratory. That is, various regions of the pJM1 plasmid are deleted at random and could be analyzed for the effect of the deletion on the ability of the derivative strain to sequester iron. Restriction endonuclease analysis of the pJM1::Tn1 derivitive plasmids has allowed me to map the regions important in iron sequestration to BamHI fragment 1 and possibly including the bordering fragments BamHI fragments 5 and 6. Further studies would complement this analysis by generating more pJM1::Tn1 insertions and insertion/deletion plasmids for a possibility of finding regulatory mutations, that is, mutations that allow expression of the iron uptake system under iron replete conditions. An analysis of this type along with molecular cloning experiments should demonstrate if the iron uptake components are plasmid encoded or simply mediated or regulated by this virulence plasmid.

A preliminary investigation into the siderophore activity mediated by the pJM1 plasmid demonstrated that this activity was not able to reverse growth inhibition due to iron stress in some other Vibrio species, nor could iron-limited culture supernatants from these other Vibrio species reverse the growth inhibited iron-stressed iuc mutant V. anguillarum 775::Tn1-5(pJHC-91). However, a class of isolates that are highly virulent but do not carry a pJM1 plasmid; that is, are plasmidless, can reverse the growth inhibition of V. anguillarum iuc and iuc, iut mutants. This cross-feeding effect needs to be further investigated to understand what type of cross-feeding substance was able to support growth of iuc and iuc, iut mutant derivitives of V.

anguillarum 775 while another substance only supports the growth of V. anguillarum iuc mutants (Appendix A).

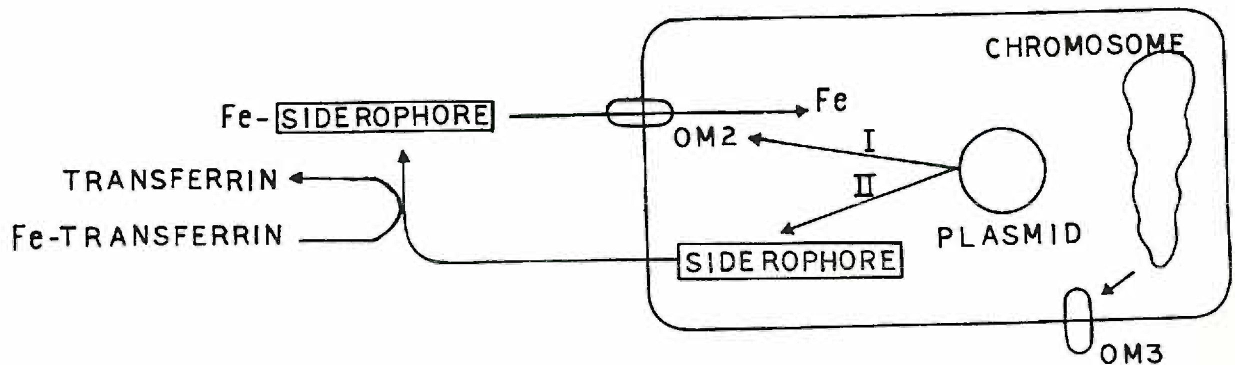
Once the pJM1 area involved with iron uptake in V. anguillarum 775 was determined using Tn1 mutation analysis, I compared this region with the iron uptake area of the pColV-K30 plasmid (Walter et al., 1984). Physiologic studies showed no apparent homology of function or DNA sequence as reported in section Paper 2 of this thesis. A continued analysis using the exact regions involved in iron uptake may provide more information on the DNA hybridization comparison. One can project the comparison with the pJM1 iron uptake regions and other Vibrio systems, Shigella systems, and Neisseria systems to mention a few.

Figure 1 of this section schematically illustrates a possible model for iron sequestration in V. anguillarum 775. Under conditions of iron stress (< 1 μM iron) V. anguillarum 775 (pJM1) produces an activity that acts as a siderophore. This compound was able to obtain iron from the iron chelators, EDDA and transferrin, and bring the 'iron-laden' complex back to the cell to be internalized (either the iron or iron complex) by a receptor activity. This receptor activity may involve a low-iron inducible protein designed OM2; however, further studies are needed to prove this connection.

Finally, the information obtainable when DNA sequencing analysis is complemented with physiological studies is vast. The further understanding of this iron sequestration system in V. anguillarum 775 mediated by the pJM1 virulence plasmid will require the combined efforts of a functional analysis (physiologic assays) and a structural analysis (DNA sequencing) (Appendix B). Moreover, the chromosomal involvement with this iron uptake system has not really been explored except for the

finding of an outer membrane chromosomally encoded protein OM3 which appears to be induced under iron limitation. This analysis will shed still further insight into the intricacies involved with the virulence factor, iron sequestration, in the marine fish pathogen, *V. anguillarum* 775.

Figure 1. High virulence *Vibrio anguillarum* schematic. This drawing represents a model for the plasmid mediated iron-sequestration system in *V. anguillarum* 775.


HIGH VIRULENCE Vibrio anguillarum
CHROMOSOME
Fe-SIDEROPHORE
Fe
OM2
I
II
TRANSFERRIN
PLASMID
Fe-TRANSFERRIN
SIDEROPHORE
OM3

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